University of Alberta

Hypothermia and Energy Substrate Metabolism in the Heart.

By

Darrell David Belke ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Pharmacology

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Darrell D. Belke

13556-126 st.
Edmonton, Alberta, Canada

Date: Jan 20, 1997

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate studies and Research for acceptance, a thesis entitled **Hypothermia and Energy Substrate Metabolism in the Heart** submitted by **Darrell David Belke** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

Professor Gary D. Lopaschuk

Professor Alexander S. Clanachan

Professor Lawrence C. H. Wang

William F. Dryden
Professor William F. Dryden,

Professor Robert O. Ryan

William W. Winder
Professor William W. Winder

Date: Jan 20, 1997

Dedication

To those scientists who have gone before, and those who will come after.

Abstract

This thesis tested the hypothesis that fatty acids depress glucose metabolism, specifically glucose oxidation, during hypothermia and rewarming and that this effect depresses the recovery of function following rewarming. Cardiac function and energy substrate metabolism were examined in isolated working rat hearts perfused with or without high concentrations of fatty acids (1.2 mM palmitate), and under conditions that stimulate glucose oxidation in the presence of high concentrations of fatty acids. Metabolism and function in hearts from rats (cold-sensitive species) were compared with hearts from Richardson's ground squirrels (cold-tolerant species). It was determined that: 1) perfusing hypothermic (15° C) rat hearts with 1.2 mM palmitate depressed glucose oxidation and was associated with a poorer recovery of function following rewarming, 2) perfusing hypothermic ground squirrel hearts with 1.2 mM palmitate does not lead to a depression in function following rewarming, 3) ground squirrel hearts are more efficient than rat hearts at converting energy into work at low temperatures; however, in rat hearts, cardiac efficiency under hypothermic conditions does not directly affect the recovery of function following rewarming, 4) stimulating glucose oxidation in rat hearts by increasing perfusate Ca^{2+} concentration or by the use of dichloroacetate is beneficial in improving the recovery of function following rewarming, 5) although the stimulation of glucose oxidation is important in reducing H^+ production under pathological conditions such as ischemia-reperfusion, H^+ production does not play a major role in mediating the detrimental effect observed following hypothermia-rewarming. This suggests that some other aspect of glucose oxidation plays a role in mediating this effect, possibly the pyruvate dehydrogenase complex activity and its role in regulating the coupling between energy production and demand, 6) the activity of acetyl-

CoA carboxlylase appears to play a role in regulating the balance of carbohydrate and fatty acid oxidation in hypothermic hearts. These results support the hypothesis that fatty acids suppress glucose oxidation during hypothermia and rewarming and that the stimulation of glucose oxidation improves the recovery of function following rewarming.

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Abbreviations

ACC: acetyl-CoA carboxylase
ADP: adenosine diphosphate
AMP: adenosine monophosphate
AO: aorta
Atm. Press.: atmospheric pressure
ATP: adenosine triphosphate
BSA: bovine serum albumin
CF: coronary flow
CO_2: carbon dioxide
CoA: co-enzyme A
CP: cardiac power
CPT 1: Carnitine palmitolytransferase 1
CPT 2: Carnitine palmitolytransferase 2
DCA: dichloroacetate
EDTA: etylenediamine-tetraacetic acid
EGTA: ethylene glycol-bis(b-aminoethyl ether)N,N,N',N'-tetraacetic acid
FADH: flavin adenine dinucleotide (reduced)
G: Gibb's free energy
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
HK: hexokinase
HPLC: high pressure liquid chromatography
LA: left atrium
LV: left ventricle
LDH: lactate dehydrogenase
MOPS: 3-[N-morpholino]propane sulfonic acid
NADH: nicotinamide adenine dinucleotide (reduced)
Na-Ca exch.: sodium-calcium exchanger
Na-H exch.: sodium-hydrogen exchanger
O_2: oxygen
PA: pulmonary artery
PCA: perchloric acid
PDC: phosphate dehydrogenase complex
PDH kinase: phosphate dehydrogenase kinase
PEG: polyethylene glycol
PFK: phosphofructokinase
PBS: phosphate buffered saline
PMSF: phenylmethylsulfonyl floride
PSP: peak systolic pressure
PV: pulmonary vein
R: gas constant
RA: right atrium
RV: right ventricle
SDS-PAGE: sodium dodecylsulfate - polyacrylamide gel electrophoresis
T: temperature
TCA cycle: tricarboxylic acid cycle
Tris: Tris(hydroxymethyl)aminomethane

Chapter I

Introduction

Since its introduction to cardiac surgery more than 40 years ago (Bigelow et al., 1950) hypothermia is now routinely used as a means of protecting the heart from the effects of prolonged ischemia. It is used clinically in conjunction with cardioplegia to produce a quiescent heart in which the expenditure of energy is dramatically reduced. Several studies have documented the beneficial effects of hypothermia in preserving intracellular ATP and creatine phosphate levels, and in reducing the production of lactate during extended periods of ischemia (Doring et al., 1976; Hearse et al., 1977; Noguchi et al., 1991; Stringham et al., 1992; Owen et al., 1993). To date, intracellular levels of high-energy phosphates and metabolites, such as lactate, have been used as an endpoint indicating protection of the myocardium, and hence its ability to recover function upon reperfusion and rewarming. However, it is now clear that the dynamics of energy metabolism (production and utilization) and not the static levels of metabolites are more indicative of functional recovery following an ischemic episode (Neely and Grotyohann, 1984; Opie, 1992a). Moreover, not only does energy production play a role in determining the recovery of mechanical function following ischemia, but the source of energy substrate is an important factor (Lopaschuk et al., 1994; Opie, 1992a). In addition, energy substrate metabolism is also an important determinant of mechanical function in hearts under pathological conditions such as diabetes or myocardial hypertrophy (Lopaschuk et al., 1994). While considerable attention has been focused on energy metabolism following ischemia (see Lopaschuk et al., 1994 for review), little is known about the relationship between energy substrate metabolism and the recovery of function following hypothermia and rewarming despite the fact that hypothermia affects both heart function and energy metabolism (Tveita et al., 1994; Hung et al., 1972; Shipp et al., 1965).

1) Heart Metabolism

1.1) Overview

The heart is omnivorous, capable of metabolizing a variety of substrates (both endogenously and exogenously derived) depending on energy needs and availability within the plasma; a feature that likely evolved to ensure an adequate supply of energy to be converted into mechanical work (Neely and Morgan, 1974, Taegtmeyer, 1994). Under aerobic conditions, substrate utilization by the heart is primarily determined by circulating concentrations of carbohydrates (glucose, lactate, pyruvate) and fatty acids, as well as the level of work being performed by the heart (0pie, 1991). The primary source of carbohydrate used by the heart is glucose, although depending on plasma concentrations lactate can become a major source of energy. Under normal resting conditions the bulk of ATP production is derived from the oxidation of fatty acids, with the contribution of glucose oxidation to energy production increasing during an increase in cardiac work. Even in the absence of exogenously supplied fatty acids the heart is still able to obtain at least 40% of its ATP from endogenous triacylglycerol stores (Saddik and Lopaschuk, 1991). Under normal physiological conditions exogenous fatty acids are available to the heart, and the contribution from endogenous triacylglycerol decreases to 10%. Thus, under most conditions the heart relies on the oxidation of exogenously supplied fatty acids as a critical source of energy for normal heart function.

The contribution of glucose and other carbohydrates to overall oxidative metabolism is dependent upon the availability of the substrates relative to non-carbohydrate sources (Opie, 1991), however, these substrates do not constitute the major source of energy production under normal physiological conditions. Although glucose and other carbohydrates do not supply the bulk of acetyl-CoA to the mitochondria for oxidative metabolism under normal conditions, a decrease in the contribution of carbohydrates to overall oxidative metabolism by only a few percent under pathological conditions such as ischemia-reperfusion can adversely affect heart function (Lopaschuk and Saddik, 1992). In addition, pyruvate obtained from carbohydrate metabolism is an important source for the anapleurotic generation of TCA cycle intermediates for oxidative metabolism (Taegtmeyer, 1994). Thus although carbohydrates provide only a minor contribution to overall oxidative metabolism, they play a critical role in maintaining oxidative metabolism and normal heart function.

In contrast to oxidative metabolism, ATP derived from glycolysis contributes only a small fraction (< 5%) to the total energy production of the heart under normal physiological conditions. While the contribution of glycolytically derived ATP to normal heart function is debatable, under pathological conditions where oxidative metabolism is limited (e.g. angina), glycolytically derived ATP becomes critical for maintaining myocyte integrity. Under these conditions (low-flow ischemia), glycolytic rate increases dramatically, and glycogen is mobilized to provide a carbohydrate source for glycolysis. In contrast, glycolytically derived ATP is detrimental under conditions where no coronary flow is available (no-flow ischemia) to remove the end products of glycolysis (lactate, H^+). Under these conditions, the increase in glycolysis is detrimental to heart function once flow is restored (reperfusion injury). Thus, energy substrate metabolism is dependent on substrate availability, and can be beneficial or detrimental depending on pathophysiological conditions.

1.2) Control of Substrate Metabolism.

1.2.1) Glycolysis

Glucose entering the myocyte is phosphorylated by hexokinase, following which it can either be stored as glycogen, enter the pentose phosphate shunt, or enter the glycolytic pathway for catabolism to pyruvic acid (Figure I.1). The first part of the glycolytic pathway involves the expenditure of ATP to break glucose 6-phosphate into the trioses dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Control is mediated by phosphofructokinase (PFK) (Figure I.1), an enzyme allosterically regulated by fructose 2,6-bisphosphate, ADP and AMP (which increase activity), and by ATP and citrate (which decrease activity). Although in many cell types fatty acids are able to inhibit glycolysis through an increase in cytosolic citrate levels, the extent to which this occurs in myocardial tissue is unknown (Neely and Morgan, 1974). PFK is also sensitive to changes in intracellular pH, with a build up of H^+ ions decreasing enzyme activity. The second part of glycolysis involves the ATP and NADH generating conversion of glyceraldehyde 3-phosphate into pyruvate. Control is mediated by glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which can be inhibited by an increase in tissue levels of lactate and NADH (Figure I.1) (Opie, 1991). Glycolysis leads to a net generation of 2 pyruvate, 2 ATP and 2 NADH from glucose.

Glucose + 2 ADP + 2 Pi + 2 NAD^+ ---> 2 pyruvate + 2 ATP + 2 NADH

Even under normal physiological conditions more pyruvate is produced by the glycolytic pathway than can be oxidized within the mitochondria, as a result pyruvate is both oxidized to and reduced to lactate. This uncoupling of glycolysis and glucose oxidation results in a ratio of glucose passing through glycolysis to that being fully oxidized which is frequently greater than 1.

1.2.2) Glucose Oxidation

Pyruvate conversion to acetyl-CoA and subsequent entry into the TCA cycle is governed by the activity of the pyruvate dehydrogenase complex (PDC). PDH kinase and phosphatase regulate PDC activity, with the phosphorylated enzyme being inactivated (Figure I.2) (Patel and Roche, 1990). The kinase is activated by acetyl-CoA and NADH and inhibited by high concentrations of pyruvate (>100 μM), ADP, free CoA and NAD^+. The phosphatase is activated by Ca^{2+} and insulin and is inhibited by NADH. The activity of the phosphatase is dependent on Mg^{2+}, and the main effect of increasing intramitochondrial Ca^{2+} is to decrease the K_m of the phosphatase for Mg^{2+} leading to its activation (McCormack et al., 1990). As a result, carbohydrate oxidation is controlled by substrate supply as well as the redox state of the mitochondria. An increase in pyruvate availability or a decrease in NADH (e.g. high energy demand) will stimulate the activity of PDC. Pharmacological agents such as dichloroacetate (DCA) can activate PDC by inactivating PDH kinase, leaving the action of the phosphatase unopposed (Stacpoole, 1989). Under ideal conditions, the oxidation of pyruvate within the TCA cycle leads to the formation of 15 ATP.

1.2.3) Fatty Acid Oxidation

Fatty acids for oxidative metabolism are derived from the plasma as free fatty acids (bound to albumin) or from lipoprotein particles (chylomicron and very low density lipoprotein) by the action of lipoprotein lipase. Once in the cells, free fatty acids associate with fatty acid binding proteins (FABPs) which facilitate their movement through the hydrophilic environment of the cytoplasm. Acyl-CoA synthase converts fatty acids into CoA thioesters (Figure I.3). Carnitine palmitolytransferase 1 (CPT 1), catalyzes the conversion of long chain acyl-CoA into long chain acylcarnitine for transport across the mitochondrial membrane. As CPT 1 represents the first step in committing fatty acids to β-oxidation it is a key regulatory enzyme in controlling the fate of fatty acids for oxidation (Sugden and Holness, 1994). Malonyl-CoA, a product of acetyl-CoA carboxylase (ACC), can inhibit the action of CPT 1 in the heart (K_i approx. 50 nM) (Lopaschuk and Gamble, 1994). Long chain acylcarnitine produced by CPT 1 is transported across the inner mitochondrial membrane by carnitine acyltranslocase and reconverted back into long chain acyl-CoA by CPT 2 in the mitochondrial matrix. Acyl-CoA then enters the β-oxidation pathway to produce acetyl-CoA. Within the mitochondria, the enzymes involved in β-oxidation are not allosterically regulated. However, 3-ketoacyl-CoA thiolase, the final step in the β-oxidation cycle is regulated by the availability of free CoA, so that an increase in acetyl-CoA/CoA ratio slows the rate of β-oxidation (Schulz, 1994). A build-up of NADH also inhibits β-hydroxyacyl-CoA dehydrogenase by depleting available NAD^+, while an increase in succinyl dehydrogenase activity inhibits acyl-CoA dehydrogenase by competing for FAD (Neely and Morgan, 1974). Under ideal conditions, the oxidation of 1 mol of palmitate (16:0) should yield 129 mol of ATP.

1.2.4) Reciprocal Control of Carbohydrate and Fatty Acid Oxidation

In addition to controlling the entry of carbohydrates and fatty acids into the TCA cycle, PDC and CPT 1 are targets for the different pathways to exert reciprocal control over substrate entry into the TCA cycle (Randle et al., 1994; Sugden and Holness, 1994). When fatty acids are the dominant substrate, a rise in the acetyl-CoA/CoA ratio leads to an activation of PDH kinase and phosphorylation of PDC to the inactive form (Figure I.4) (Patel and Roche, 1990).

Conversely, an increase in carbohydrate metabolism leads to an increased production of malonyl-CoA from ACC and an inhibition of CPT 1 (Figure I.5). Under this scheme, acetyl-CoA from PDC is preferentially available for export from the mitochondria (as acetylcarnitine) via the action of a carnitine acetyltransferase, acetylcarnitine translocase system (Lysiak et al., 1988). Once in the cytoplasm, acetylcarnitine is reconverted to acetyl-CoA where it is subsequently converted into malonyl-CoA by ACC. Direct stimulation of PDC using DCA increases the contribution of carbohydrate to oxidative metabolism at the expense of fatty acid oxidation (Saddik et al., 1993). Similarly, indirect stimulation of PDC through carnitine loading and its subsequent activation of the carnitine acetyltransferase, acetylcarnitine translocase system, also increases the contribution of carbohydrate to oxidative metabolism (Broderick et al., 1993). As a result of the reciprocal interactions between carbohydrates and fatty acids, the pattern of oxidative metabolism is altered by changes in the oxidation rates of individual substrates.

1.3) Oxidative Phosphorylation

Energy substrates are converted into ATP through the oxidation of NADH and FADH derived from substrate metabolism, and the movement of electrons down the electron transport chain (Figure I.6). The movement of electrons down the transport chain provides the energy to pump protons across the inner mitochondrial membrane. ATP synthesis occurs when the protons re-enter the mitochondrial matrix through the ATP synthase. The amount of ATP derived from the oxidation of NADH and FADH is debatable; however, as electrons derived from FADH enter the electron transport chain at site II instead of site I, leading to the translocation of fewer protons, less ATP is derived from the oxidation of FADH than NADH. Typically, values quoted for ATP production /

O_2 consumed (P/O) is 3 for NADH and 2 for FADH (Opie, 1991); however, these values are ideal, and are not achieved under experimental conditions (Lee et al., 1996; Hinkle et al., 1991). The reason for the non-ideal P/O ratios may be explained by proton leakage across the inner mitochondrial membrane into the matrix (not associated with ATP production). It may also result from a failure of protons to be pumped against the proton gradient when the gradient across the inner-mitochondrial membrane is steep (slippage) (Brand et al., 1994; Canton et al., 1995). In muscle tissue, the leakage of protons may account for up to 52% of O_2 consumption at rest (Brand et al., 1994). In addition, ion exchangers in the mitochondrial membrane may use the energy of the proton gradient for regulating ion concentrations (Ca^{2+}, Na^{+}) within the mitochondrial matrix (Opie, 1991). The P/O ratio has also been shown to be affected by thyroid status (Harper and Brand, 1994) and membrane fluidity (Rottenberg, 1978; Dufour et al., 1996). As a result, the total ATP production from energy substrate metabolism is unknown due to a variable stoichiometry between the level of energy substrate metabolized and the level of ATP produced. It is assumed that this inefficiency in ATP production occurs regardless of the source of acetyl-CoA for the TCA cycle (e.g. carbohydrate or fatty acid).

The amount of O_2 required to produce ATP from fatty acids is higher than that required for carbohydrates. As a result, to make equimolar amounts of ATP from fatty acids than glucose requires 12% more O_2 (Opie, 1991). This is due, in part, to the reduction of FAD to FADH in the β-oxidation pathway, and the fact that FADH contributes electrons to the electron transport chain further along the pathway than NADH. As a result, the extent of fatty acid oxidation under conditions where oxygen may be limiting can determine the amount of ATP synthesized.

1.4) Substrate Metabolism and Heart Function.

The activity of individual metabolic pathways can affect heart function. As a result, it is desirable in experimental studies to have a good index of the energy status of the heart. Direct measurement of ATP production rates, and the source of this ATP production, provides a better indicator of metabolism than simply measuring static high-energy phosphate (ATP, creatine phosphate) levels (Opie, 1992b).

Under normal physiological conditions glycolysis contributes little to overall ATP production. However, under pathological conditions, glycolytically derived ATP becomes an important source of energy when oxidative metabolism is depressed (e.g. as a result of low flow ischemia, or hypoxia). Weiss and Hiltbrand (1985) have suggested that sarcolemmal membrane ion gradients are maintained by ATP derived from glycolysis. Several studies have indicated that glycolytically derived ATP may be preferentially used by enzymes involved in ion homeostasis such as sarcolemmal Na^+-K^+ ATPase, sarcolemmal Ca^{2+} ATPase, and the sarcoplasmic reticulum Ca^{2+} ATPase (Jeremy et al., 1992; Kristensen 1993; Glitsch and Tappe, 1993; Xu et al., 1995). These studies suggest that glycolytically derived ATP plays a more prominent role in the maintenance of ion homeostasis than ATP derived from oxidative metabolism.

In contrast, increased glycolytic flux during total (no flow) ischemia can be detrimental due to the development of tissue acidosis. This occurs as the result of the hydrolysis of ATP derived from glycolysis (Dennis et al., 1991). This increase in tissue acidosis results in the development of a Ca^{2+} overload upon reperfusion due to the actions of the Na^+-H^+ and Na^+-Ca^{2+} exchangers (Figure I.7) (Tani and

Neely, 1989). Although increasing glycolytic flux may be beneficial or deleterious depending on the type and degree of insult, complete inhibition of glycolysis even in the presence of adequate oxidative metabolism can also lead to myocardial dysfunction (Kusuoka and Marban, 1994). Thus, although glycolysis contributes little to overall ATP production in myocytes it may provide a specific pool of ATP required for ion homeostasis; however, over-activity or inhibition of this pathway can be detrimental depending on the pathological conditions.

Although the bulk of ATP for cardiac work is derived from oxidative metabolism, an over reliance on fatty acid oxidation as a source of energy in pathological states (e.g. diabetes, myocardial infarction) is detrimental to heart function. Plasma fatty acid levels can increase dramatically in under pathological conditions so that the heart is exposed to fatty acid concentrations 3-5 times that observed in normal healthy individuals (Lopaschuk et al., 1994b; Teoh et al., 1988b, Rodrigues et al., 1995). Elevated plasma fatty acid levels have been associated with an O_2 wasting effect, decreasing myocardial efficiency, and a high incidence in the development of arrhythmias (Oliver and Opie, 1994). Initial studies suggested that an increase in fatty acid metabolites such as acylcarnitine mediated the deleterious effects of fatty acids by acting as a detergent to disrupt membrane function leading to an ion imbalance and Ca^{2+} overload (Corr et al., 1984). However, it is uncertain whether sufficient amounts of fatty acid metabolites can accumulate to disrupt membrane function under pathological conditions (Lamers et al., 1987). Similarly, the protective effects of CPT 1 inhibitors following ischemia-reperfusion have been shown to occur independently of changes in long chain acylcarnitine levels (Lopaschuk et al., 1988; Heathers et al., 1993).

While the role of fatty acid metabolites such as acylcarnitine in mediating myocardial dysfunction is uncertain, increasing plasma fatty acid levels have been implicated in playing a role in mediating myocardial dysfunction (Oliver and Opie, 1994). This effect is closely associated with reperfusion following ischemia, where the myocardium shows a greater reliance on fatty acids as a source of energy. Increased rates of fatty acid oxidation may occur through an increase in fatty acid substrate supply, or as the result of an intervention leading to increased CPT 1 activity (Liedtke et al., 1988; Mickle et al., 1986; Lopaschuk and Saddik, 1992; Kudo et al., 1995). That an increased reliance on fatty acids as an energy source is detrimental to heart function is also supported by observations that increasing glucose oxidation is beneficial to the post ischemic recovery of cardiac function. Direct stimulation of glucose oxidation by dichloroacetate leads to an improvement in mechanical function of the heart following ischemia reperfusion (McVeigh and Lopaschuk, 1990, Lewandowski and White, 1995). Similarly, the stimulation of glucose oxidation and a reduction in fatty acid oxidation by ranolazine is beneficial to the recovery of heart function following ischemia-reperfusion (McCormack et al., 1996). In addition, indirect stimulation of glucose oxidation by CPT 1 inhibitors such as etomoxir (Lopaschuk et al., 1988), or by carnitine loading of the heart (Broderick et al., 1993) has been shown to be beneficial in improving functional recovery following ischemia-reperfusion.

The exact mechanism by which decreasing fatty acid oxidation and increasing glucose oxidation improves cardiac function is unknown. An increase in plasma fatty acid level is associated with an increase in O_2 consumption relative to cardiac function (O_2 wasting effect) (Vik-Mo and Mjos, 1981). The increased O_2 consumption observed with fatty acids under normal physiological conditions is not associated with an increase in ATP utilization within the heart (Hutter et al., 1985, Kingsley-Hickman et al., 1990), implying a direct effect of fatty acids on O_2 consumption within the heart. Improving carbohydrate metabolism decreases the O_2 demand of the heart as the respiratory quotient for carbohydrate metabolism is 1.0 while that of fatty acid oxidation is 0.7 (Opie, 1991). This effect is thought to improve ATP production under conditions where O_2 availability is limited.

Another hypothesis is that a reduction in glucose oxidation caused by increased fatty acid oxidation leads to an increase in H^+ production. The hydrolysis of ATP derived from glycolysis linked to lactate production yields a net production of 2 H^+ (Dennis et al., 1991). In contrast, the hydrolysis of ATP derived from glucose passing through glycolysis to pyruvate and then being completely oxidized yields a net production of 0 H^+. An increased H^+ production can lead to Ca^{2+} overload through the actions of the Na^+-H^+ and Na^+-Ca^{2+} exchangers (Figure I.6) (Tani and Neely, 1989). The stimulation of glucose oxidation by DCA following ischemia-reperfusion leads to a reduction in the uncoupling of glycolysis and glucose oxidation and hence the extent of H^+ production (Lopaschuk et al., 1993). This is beneficial to the recovery of heart function and to improving the efficiency of converting energy into mechanical work. By avoiding the intracellular accumulation of Ca^{2+}, these hearts avoid the increased energy expenditure caused by the rise in intracellular Ca^{2+}.

Other theories proposed to explain the beneficial effects of increasing PDC activity are related to an enhancement of the cytosolic phosphorylation potential (Zweier and Jacobus, 1987; Bunger et al., 1989). A decrease in phosphorylation potential is thought to play a role in mediating contractile failure by decreasing the activity of energy driven reactions such as SR Ca^{2+}-uptake and myofibrillar ATPase (Kammermeier, et al., 1982; Zweier and Jacobus, 1987, Mallet and

Bunger, 1994). In support of this concept, pyruvate has been shown to enhance function in normal and post-ischemic hearts by improving the cytosolic phosphorylation potential (Bunger et al., 1989). Although the mechanism relating this effect is uncertain, it is thought that an increase in NADH production as a result of stimulating PDC allows energy production (ATP synthesis) to become more responsive to energy demand (Zimmer et al., 1989; From et al., 1990). Under conditions where energy demand is high (i.e. increased ADP levels), energy production can only meet demand if sufficient NADH is produced from substrate metabolism to allow ATP formation to occur unhindered. When NADH production is limiting, the mitochondrion is no longer responsive to energy demand in the form of ADP availability. Improving PDC activity is thought to increase NADH availability and make energy production within the hearts more responsive to energy demand. ATP production from glycolysis also affects cytosolic phosphorylation potential; however, an increase in PDC activity seems to have a more predominant effect in altering cytosolic phosphorylation potential (Scholz et al., 1995).

This mechanism also explains how the heart is capable of responding to increased energy demand in response to positive inotropy. A rise in intramitochondrial Ca^{2+} is known to increase NADH levels by stimulation of PDC as well as dehydrogenases within the TCA cycle (McCormack et al., 1990). This has the effect of making ATP synthesis more responsive to energy demand during an increase in the work level being performed by the heart.

2) Hypothermia

Although energy metabolism, and the conditions and factors that influence the various metabolic pathways have been examined extensively under normothermic conditions, little is known about the interactions between hypothermia and energy metabolism. Through its effects on various enzymes and metabolic pathways, hypothermia is capable of altering cellular physiology in both the resting state and the response to specific perturbations. For example, isoproterenol causes a positive inotropic effect in papillary muscles at high temperatures, but causes a negative inotropic effect at 15° C (Senturia et al., 1986). The altered physiological state is the result of a depression in the rate at which enzymatic reactions occur and the fact that hypothermia does not effect all enzymes and metabolic pathways evenly. Regarding energy metabolism, hypothermia generally reduces metabolic demand by 2 fold for every 10°C change, but may not apply its effects evenly over the various pathways. Hypothermia can affect the different metabolic pathways depending on the temperature sensitivity of the rate limiting enzymes in the pathway. Hypothermia can also alter cellular physiology so that allosteric control of the enzymes governing metabolism is lost. The pattern of energy expenditure may also change under hypothermic conditions so that more energy is required for non-work related processes.

2.1) Physical Effects of Hypothermia

Temperature affects most aspects of cellular biochemistry and physiology, although its role is often overlooked due to the homeothermic nature of most mammals. Hypothermia affects the physical properties of cells and their environment (e.g., hypothermia affects the solubility of gasses in solution, the distribution of ions across a membrane, etc.). Hypothermia slows the rate of biochemical reactions by decreasing the proportion of activated enzyme-substrate complexes with sufficient levels of kinetic energy to form a product according to the Maxwell-Bolzmann distribution (Cossins and Bowler, 1987). The free energy change of a reaction ΔG (Gibb's free energy), which determines if the reaction is thermodynamically favorable, is also influenced by temperature through the form of the equation:

$$\Delta G = \Delta G^{o} + RT\ln([\text{products}] / [\text{reactants}])$$

where ΔG^{o} is the standard free-energy change of a reaction, R is the gas constant and T is temperature (K). This equation describes the free energy available from high energy phosphates (such as ATP and creatine phosphate) which is available to drive thermodynamically unfavorable reactions such as the movement of ions against a gradient. Temperature can affect biochemical reactions by reducing the free energy available to drive reactions forward, or to make them more thermodynamically unfavorable by making ΔG more positive. As energy requiring reactions can be sensitive to changes in the free energy of ATP hydrolysis, this can have a large impact on myocyte physiology (Kammermeier et al., 1982; Zweier and Jacobus, 1987; Mallet and Bunger, 1994). As a result, the effects of temperature on the heart are complex, affecting every aspect of myocardial physiology and biochemistry.

In addition to the general effects of temperature on enzymatic reaction rates, the activity of some enzyme reactions utilizing ATP (especially membrane bound enzymes) is further reduced at low temperatures by temperature-induced changes in their physical environment. This change manifests itself as an increase in the energy required to form the active complex necessary for the reaction to occur. Therefore, the beneficial effects of hypothermia in preserving ATP levels are related to its ability to decrease reaction rates by: 1) decreasing the proportion of active complexes with sufficient kinetic energy to form products, 2) by altering the free energy of a reaction to make it less thermodynamically favorable, 3) by altering the environment of ATP utilizing enzymes to make them less efficient catalysts. As a result of hypothermia, the rate of ATP utilization is decreased, however, this change occurs at the expense of the myocytes ability to maintain a normal cellular physiology.

2.2) Hypothermia and Myocardial Protection

The use of hypothermia as component of myocardial protection was introduced by Bigelow et al (1950) over 40 years ago. It is now routinely used to depress metabolic demand during heart surgery and transplantation. Hypothermia combined with high K^+ cardioplegia, to provide a quiescent heart, forms the basic tenet of myocardial protection, which is "that a reduction in metabolic demand preserving energy levels during ischemia is correlated with a reduction in the level of post-operative impairment". In support of this tenet, numerous studies have documented the beneficial role of hypothermia in preserving intracellular ATP and creatine phosphate levels during periods of prolonged ischemia (Doring et al., 1976; Hearse et al., 1977; Noguchi et al., 1991; Stringham et al., 1992, Owen et al., 1993). However, as with ischemia-reperfusion, the level of high energy phosphates at the end of hypothermic preservation is not always correlated with the recovery of function (Munfahk et al., 1991).

Although hypothermia preserves myocyte structural integrity and cardiac function better than normothermic ischemia, hypothermia itself affects heart function upon rewarming even in the absence of noticeable indicators of ischemia (i.e. depressed ATP and creatine phosphate levels). Recent clinical studies investigating the effects of cardioplegic temperature on post-operative cardiac function have suggested that cold cardioplegia is more detrimental than warm cardioplegia. This is demonstrated by a better recovery of function and a reduction in creatine kinase release in heart treated with warm cardioplegia rather than cold cardioplegia (Yau et al., 1993; Mauney and Kron 1995). Even under conditions where ischemia is avoided by the continuous perfusion of cardioplegic solution, hypothermia leads to disruptive ultrastructural changes within the myocyte that can lead to myocardial dysfunction upon rewarming (Ferrera et al., 1994).

The poor recovery of function following hypothermia-rewarming may be linked to a loss in activator Ca^{2+} to stimulate contraction, and changes in membrane integrity. Hess et al (1981) has demonstrated that subjecting hearts to global hypothermic ischemia results in a reduction in sarcoplasmic reticulum (SR) function. Previous studies have attributed the reduction in SR function to ischemia (Krause et al., 1989). Recent studies have indicated that cold cardioplegia even in the absence of ischemia can lead to a reduction in SR function and a loss of intracellular Ca^{2+} homeostasis (Liu et al., 1993, 1994). Steigen et al (1992) have shown that hypothermia leads to structural changes within the sarcolemmal phospholipid bilayer, leaving it susceptible to phospholipase attack following rewarming. This suggests that membrane structure is altered following hypothermia-rewarming. Thus, although hypothermia is generally associated with protection of the myocardium from the adverse effects of ischemia in a clinical setting, hypothermia itself, even in the absence of damage derived from ischemia, can result in a disruption of myocyte integrity and physiology.

Hypothermia can also be deleterious to heart function under conditions where the heart is subjected to neither cardioplegia or ischemia. Although hypothermia reduces overall O_2 consumption in the working heart, it leads to a dissociation between O_2 consumption and work so that cardiac efficiency decreases, suggesting a greater expenditure of energy on non-work related processes such as the maintenance of trans-membrane ionic gradients (Archie and Kirklin, 1973; Tveita et al., 1994, Russ and Lee, 1965). Whole animal studies have shown that this decrease in cardiac efficiency persists during rewarming from hypothermia, leading to a reduction in cardiac output and eventually circulatory collapse and death (Tveita et al., 1994, 1996; Blair et al., 1956; Hung et al., 1972). Under these conditions, O_2 consumption becomes uncoupled from the level of work being performed, resembling the myocardial stunning observed following ischemia-reperfusion (Tvieta et al., 1994; DeMaison and Grynberg, 1994). That this decrease in efficiency occurs in the absence of hypoxia, (arterial O_2 levels are maintained above euthermic controls) and acidosis (Tvieta et al., 1996) suggests that this effect is not related to the development of ischemia or poor heart perfusion.

2.3) Hypothermia and Intracellular Ca^{2+}Homeostasis

As hypothermia decreases a cell's ability to pump ions against a gradient to a greater degree than the influx of ions down their electrochemical gradient (Willis, 1987), many of the interventions aimed at reducing hypothermic damage are related to protecting tissues against the loss of ion homeostasis. Of the potential changes in intracellular ion concentrations an increase in intracellular Ca^{2+} is the most detrimental. The importance of Ca^{2+} as an intracellular signal mediating excitation-contraction coupling, and the large transmembrane gradient across the sarcolemmal membrane, makes the loss of Ca^{2+} homeostasis detrimental to the heart. A reduction in Na^+-K^+ ATPase activity leads to a rise in intracellular Na^+ which can result in an increase in intracellular Ca^{2+} through the actions of the sarcolemmal Na^+-Ca^{2+} exchanger (Martineau-Knerr and Lieberman, 1993). Hypothermia can lead to an increase in myocardial Ca^{2+} levels, even in the absence of ischemia (Liu et al., 1991; Steigen et al., 1994). Any reduction in oxidative metabolism (i.e. hypoxia) can exacerbate Ca^{2+} accumulation under hypothermic conditions (Navas et al., 1990). As a result, efforts aimed at improving myocardial protection during hypothermic preservation have attempted to prevent or reduce the rise in intracellular Ca^{2+} (Liu et al., 1994; Robinson and Harwood, 1991; Bjornstad et al., 1994; Rebeyka et al., 1990). Unfortunately, a hypothermia-induced reduction in SR function may contribute to the Ca^{2+} overload observed following hypothermia and rewarming (Labow et al., 1993; Fukumoto et al., 1991; Liu et al., 1993, 1994). The resulting increase in the level of intracellular Ca^{2+} during hypothermia is associated with a depression in the recovery of function following rewarming (Steigen et al., 1994; Aasum and Larsen, 1995).

2.4) Comparison of Hypothermia and Ischemia on Heart Function

Although hypothermia is commonly used as protection against ischemia, both hypothermia-rewarming and ischemia-reperfusion can have similar effects on heart function. Both lead to a disruption of ion homeostasis where intracellular Na^+ and Ca^{2+} are increased (Martineau-Knerr and Lieberman, 1993; Tani and Neely, 1989). Both can lead to: a) a depression in SR function (Krause et al., 1989; Liu et al., 1994), b) the development of arrhythmias (Liu et al., 1990; Oliver and Opie, 1994), and c) to stunning and a reduction in cardiac efficiency (DeMaison and Grynberg, 1994; Tveita et al., 1994). Both hypothermia and ischemia can potentiate the deleterious effects of the other. Hypothermia has been shown to exacerbate the detrimental effects of ischemia (Ting et al., 1994). Ischemia, over a prolonged period of time, can induce a degree of damage in hypothermic hearts greater than that of hypothermia alone (Ferrera et al., 1994). Both are susceptible to variations in substrate metabolism. Exposing ischemia-reperfused hearts to high concentrations of fatty acids leads to a poor recovery of function during reperfusion (Lopaschuk et al., 1994). Under analogous conditions, exposing hypothermic hearts to high concentrations of palmitate leads to a poor recovery of function following rewarming (Mjos et al., 1991, Steigen et al., 1994). Thus, hypothermia and ischemia share a number of similar traits in disrupting myocardial physiology and depressing heart function.

2.5) Hypothermia and Metabolism

Apart from the ability of hypothermia to reduce overall metabolic demand, little is known about the direct effects of hypothermia on specific metabolic pathways in the heart. Despite the lack of knowledge in this area, attempts have been made to prolong myocardial viability by stimulating oxidative metabolism through an increase O_2 and substrate supply during hypothermic cardioplegia (Doherty et al., 1992, Teoh et al., 1988a; Ohkado A et al., 1994; Choong et al., 1993). Similarly, blood cardioplegia (4 parts blood, 1 part high K^+ cardioplegic solution) is often used in clinical settings as a means of increasing O_2 availability to the heart by improving the low O_2 solubility of normal cardioplegic solutions (Vinten-Johansen et al., 1991). The recent work by Mjos et al (1991) and Steigen et al (1994) have shown that exposing hearts to high concentrations of fatty acids during hypothermia depresses heart function following rewarming. As plasma fatty acid concentrations can increase dramatically during surgery (Lopaschuk et al., 1994b; Teoh et al., 1988b, Rodrigues et al., 1995), this practice may increase the risk of exposing the hypothermic heart to high concentrations of fatty acids which could be detrimental to heart function following rewarming. A better understanding of the interactions between energy substrate metabolism and hypothermia would be beneficial in designing strategies to improve hypothermic storage and the recovery of normal function following rewarming.

Despite growing evidence that energy substrate metabolism can affect heart function, little is known about the direct effects of hypothermia on the activity of specific energy yielding pathways. The general effects of hypothermia on energy substrate metabolism have been examined under conditions of whole body hypothermia. Animal studies have suggested that glucose metabolism is depressed to a greater extent than fatty acid metabolism during hypothermia, despite a large increase in plasma glucose levels (Hoo-Paris et al., 1988; Tveita et al., 1996). Analysis of plasma fatty acid oxidation in relation to whole animal O_2 consumption under hypothermic conditions suggests that a greater proportion of oxidative metabolism is supported by fatty acid oxidation (Jourdan, 1991). These studies indicate that the pattern of energy substrate metabolism is altered as a result of hypothermia. It is not possible to determine whether these changes represent general changes in overall metabolism, or the differential effects of hypothermia on specific tissues, due to the fact that these studies represent the effects of hypothermia on the whole animal.

Much of what is known about substrate metabolism in the hypothermic heart has been inferred from differences in measurements of arterio-venous (A-V) substrate composition (Shida et al., 1977; Teoh et al., 1988a). While many of these studies support with the observation made in whole animal studies that glucose metabolism is decreased during hypothermia, they also suggest that the utilization of other carbohydrate sources (such as pyruvate and lactate) are increased relative to their euthermic rates (Shida et al., 1977; Teoh et al., 1988a; Edwards et al., 1954; Russ and Lee, 1965). Similarly, some studies have suggested that fatty acid oxidation is increased during hypothermia as a result of decreased glucose metabolism (Russ and Lee, 1965; Shida et al., 1977, Teoh et al., 1988a). Following rewarming, other studies have suggested that glucose oxidation is depressed (Drake-Holland et al., 1984), while the oxidation of other carbohydrates such as lactate and pyruvate are increased (Teoh et al., 1988b). Adding confusion to this issue is the study of Smolenski et al (1994) which suggests that carbohydrate metabolism is depressed during the first few minutes of reperfusion following hypothermia, but increases over time so that a net increase in pyruvate utilization occurs. Other studies have also suggested that fatty acid oxidation can either be increased (Mickle et al., 1986) or decreased (Teoh et al., 1988b) following rewarming and reperfusion of the myocardium. The main problem with many of these studies is that the uptake of substrates as measured by A-V differences may not accurately reflect oxidative metabolism, as some degree of substrate storage may occur (Teoh et al., 1988a, Wisneski et al., 1985). Furthermore, these clinical studies employ cardioplegia and ischemia to varying degrees along with mild or severe hypothermia making any assessment of the individual components on metabolism difficult. In addition, most of these studies did not include any assessment of function following rewarming, so that the relationship of metabolism to mechanical function could not be assessed. Due to these factors, the effects of hypothermia and rewarming on energy substrate metabolism in the heart are uncertain.

3) Cold-tolerant Species.

In contrast to cold-sensitive mammalian species, mammalian hibernators have evolved the means of safely utilizing low body temperatures to conserve energy in cold environments. Mammalian hibernators are capable of surviving for many days at body temperatures approaching 0° C (Wang, 1989). Survival at low temperatures is dependent on maintaining physiological control over body temperature, circulation, respiration and a number of other systems, along with the ability to rewarm to 37° C without any adverse effects. Although these animals undergo a number of physiological and biochemical changes when preparing for hibernation, they possess a general resistance to the deleterious effects of hypothermia regardless of their state within the annual hibernation cycle.

3.1) Hearts of Hibernating Species

Hearts from hibernating species are able to tolerate the effects of low temperature and remain functional at temperatures approaching 0° C, whereas those from cold-sensitive rodent species generally cease functioning at temperatures between 10 and 15° C (Lyman and Blinks, 1959; Burlington and Darvish, 1988; Caprette and Senturia, 1984). While the nature of this cold tolerance is not completely understood, it is clear is that the cold tolerance of hearts from hibernating species is independent of the seasonal differences in the biochemical and biophysical properties of the heart (Caprette and Senturia, 1985, Smith and Katzung, 1966). Although many studies have suggested that the cold tolerance of hearts from hibernating species is dependent upon differences in the electrophysiological properties of cold-tolerant and cold-sensitive species (Liu et al., 1991a, Marshall and Willis, 1962), more recent work has suggested that factors such as resting membrane potential, and the properties of Ca^{2+} channels are not major contributors to cardiac dysfunction; rather, the ability to regulate intracellular Ca^{2+} appears to play a more important role (Herve et al., 1992). The ability of SR to accumulate Ca^{2+} is increased in hearts from ground squirrels in the hibernating state (Belke et al., 1991) suggesting an improved ability to regulate intracellular Ca^{2+} in this state. In addition, the activity of SR from ground squirrels in the non-hibernating state is less sensitive to inhibition by temperature than SR from rat hearts (Liu et al, 1996). This may explain the greater rise in intracellular Ca^{2+} observed in isolated myocytes from rats as compared with ground squirrels under hypothermic conditions (Liu et al., 1991b; Liu and Wang, 1993).

3.2) Myocardial Energy Metabolism in Hibernating Species.

Hibernating animals store large quantities of fat prior to entering hibernation, and are able to utilize fatty acids as a source of energy during hibernation (Galster and Morrison 1966; Suomalainen and Saarikoski, 1967). However, beyond this observation, little is known about energy substrate metabolism by tissues such as the myocardium tissue, either as an effect of temperature, or physiological state. Indirect evidence suggesting an increase in fatty acid oxidation in hearts of hibernators is obtained from studies showing an increased in carnitine content (Burlington and Shug, 1981) and electron micrographs showing an increased number of lipid droplets in the hibernating state (Burlington et al., 1972). Similarly, Brooks and Story (1992) have shown that PDC activity is decreased in ground squirrel during the hibernating state. However, despite these observations, a direct comparison of myocardial energy substrate metabolism has not made between animals in the hibernating or non-hibernating state.

Although comparisons of myocardial energy metabolism have been made between cold-tolerant (hibernating) and cold-sensitive species, these changes have been confined to differences in the level of ATP and other adenine nucleotides. Covino and Hannon (1959) were able to show that the ATP level in ground squirrel myocardium was higher than in rabbit myocardium at 15° C. However, they did not ensure that the rabbits were properly ventilated under these conditions and these results may have been influenced by the development of hypoxia in the rabbits. Burlington et al (1976) determined ATP, ADP and P_i levels in the isolated perfused hearts of rats and ground squirrels cooled to 20° C or lower. Although they observed a decrease in ATP levels in ground squirrel hearts, the "energy charge", determined as the ratio of ATP to ADP + P_i, remained constant over the temperature range, while this value decreased in rat hearts below 20° C, primarily due to an increase in ADP and P_i. The authors interpret these results as indicating that a balance between the synthesis, translocation and utilization of energy compounds exists in ground squirrel but not rat heart, suggesting that the factors determining energy metabolism differ greatly between the two species. Despite these observations, little is known about the contribution of the various metabolic pathways to energy production in cold-tolerant and cold-sensitive species under hypothermic conditions.

4) Acetyl-CoA Carboxylase Control of Fatty Acid Oxidation

Although the role of fatty acid oxidation in regulating the metabolism of carbohydrates by inhibition of PDC within the mitochondria is well understood (Randle et al., 1994), less is known about the regulation of fatty acid oxidation. Carnitine palmitoyltransferase 1 (CPT 1), catalyzes the conversion of acyl-CoA into acyl-carnitine, and controls the entry of fatty acids into the mitochondria. Under physiological conditions the action of CPT 1 is inhibited by malonyl-CoA, a product of acetyl-CoA carboxylase (ACC) (McGarry et al., 1989). ACC is the major rate limiting enzyme for fatty acid synthesis, catalyzing the carboxylation of acetyl-CoA into malonyl-CoA, a substrate for fatty acid synthase. Although the inhibition of CPT 1 by malonyl-CoA is important in preventing the simultaneous synthesis and oxidation of fatty acids in tissues capable of also synthesizing fatty acids (e.g. liver, adipose tissue), isoforms of ACC have recently been identified in tissues not involved in fatty acid synthesis (e.g. heart and skeletal muscle) (Bianchi et al., 1990, Thampy, 1989). ACC exists in a 265 kDa isoform and a 280 kDa isoform, with different amounts of the two isoforms being expressed to varying degrees within different tissues. In tissues capable of synthesizing fatty acids (e.g. white adipose) the 265 kDa isoform is predominant, while in tissues which oxidize fatty acids (e.g. muscle) the 280 kDa isoform is predominant (Bianchi et al., 1990). In those tissues capable of both fatty acid synthesis and oxidation (e.g. liver, brown adipose tissue), both isoforms are highly expressed.

In rat hearts, ACC primarily functions to regulate fatty acid oxidation (Saddik et al., 1993, Awan and Saggerson, 1993), since cardiac CPT 1 is extremely sensitive to inhibition by malonyl-CoA (IC_{50} = 30 - 80 nM) (Mills et al., 1983; McGarry et al, 1983; Cook, 1984). A good correlation between the activity of ACC, tissue levels of malonyl-CoA, and the rate of fatty acid oxidation has been observed under normal physiological conditions (Saddik et al., 1993), as well as pathological conditions such as ischemia-reperfusion (Kudo et al., 1995, 1996). These studies suggest that the regulation of ACC activity represents a potential means of controlling fatty acid oxidation under pathological conditions. Under hypothermic conditions, ACC activity may also be important in regulating fatty acid oxidation. That CPT 1 is capable of being regulated by ACC activity under hypothermic conditions has been suggested by previous studies using liver mitochondria (Zammit et al., 1984; Zammit and Corstorphine, 1985; Kolodziej and Zammit, 1990; Kashfi and Cook, 1995). In these studies, CPT 1 was more sensitive to inhibition by malonyl-CoA at lower temperatures than at normothermic temperatures. This suggests that the activity of ACC may play an important role in regulating fatty acid oxidation in the heart under hypothermic conditions.

5) Thesis Objectives

Despite the observation that alterations in myocardial metabolism of glucose and fatty acids can influence heart function under pathological conditions, and that hypothermia can adversely alter myocardial physiology and biochemistry, the energy substrate metabolism in the hypothermic heart is poorly understood. Due to the general influence of hypothermia on myocardial physiology and biochemistry, the control of energy substrate metabolism may be altered to predispose the heart to the detrimental effects of fatty acids. Measurement of energy metabolism in a cold-tolerant and cold-sensitive species should provide a useful comparison for determining the factors influencing energy metabolism during hypothermia, and how energy substrate metabolism is affected by hypothermia-rewarming.

The objective of this study is to: 1) determine what effect hypothermia-rewarming has directly on glycolysis, glucose oxidation and fatty acid oxidation in both cold-sensitive and cold-tolerant species, 2) to examine differences in substrate metabolism between cold-sensitive and cold-tolerant species to determine if any differences in substrate utilization may exist which account for the sensitivity of the two species to low temperature, 3) to examine the effects of altering glucose oxidation on the recovery of function following rewarming, and 4) to examine differences in substrate metabolism in ground squirrels between animals in the hibernating and non-hibernating state to determine what adaptations occur to alter the contribution of fatty acids and glucose to oxidative metabolism.

The studies presented in this thesis test the general hypothesis that high concentrations of fatty acids can depress glucose oxidation during hypothermia and rewarming, and that this leads to a poor recovery of function. They also determine whether improving glucose oxidation leads to a better recovery of function. Furthermore, I hypothesize that because cold tolerant species rely on fatty acids as a source of energy, hearts from these animals are generally unaffected by high concentrations of fatty acids, and that a decrease in ACC activity during hibernation allows these animals to utilize fatty acids to a greater extent.

6) Outline of Individual Studies

Chapter III: examines the affects of fatty acids on glucose metabolism in rat hearts, and tests the hypothesis that fatty acids depress glucose oxidation under hypothermic conditions.

Chapter IV: compares energy substrate metabolism in rats (cold-sensitive species) and ground squirrels (cold-tolerant species), to determine what differences in energy substrate metabolism occur between these species as a result of hypothermia-rewarming.

Chapter V: compares energy substrate metabolism in rats and ground squirrel subjected to hypothermia under different Ca^{2+} concentrations, and tests the hypothesis that a stimulation of glucose metabolism by Ca^{2+} is beneficial to the recovery of function during rewarming in cold-sensitive species but has no effect in cold-tolerant species.

Chapter VI: examines the effects of treating rat hearts with dichloroacetate to stimulate glucose oxidation on the recovery of function following rewarming, and tests the hypothesis that direct stimulation of glucose oxidation is beneficial to the recovery of function during rewarming.

Chapter VII: examines glucose and fatty acid oxidation in the hearts of ground squirrels in the hibernating and the non-hibernating state and tests the hypothesis that changes in acetyl-CoA carboxylase activity mediates an increased reliance on fatty acids as a source of energy during the hibernating state.

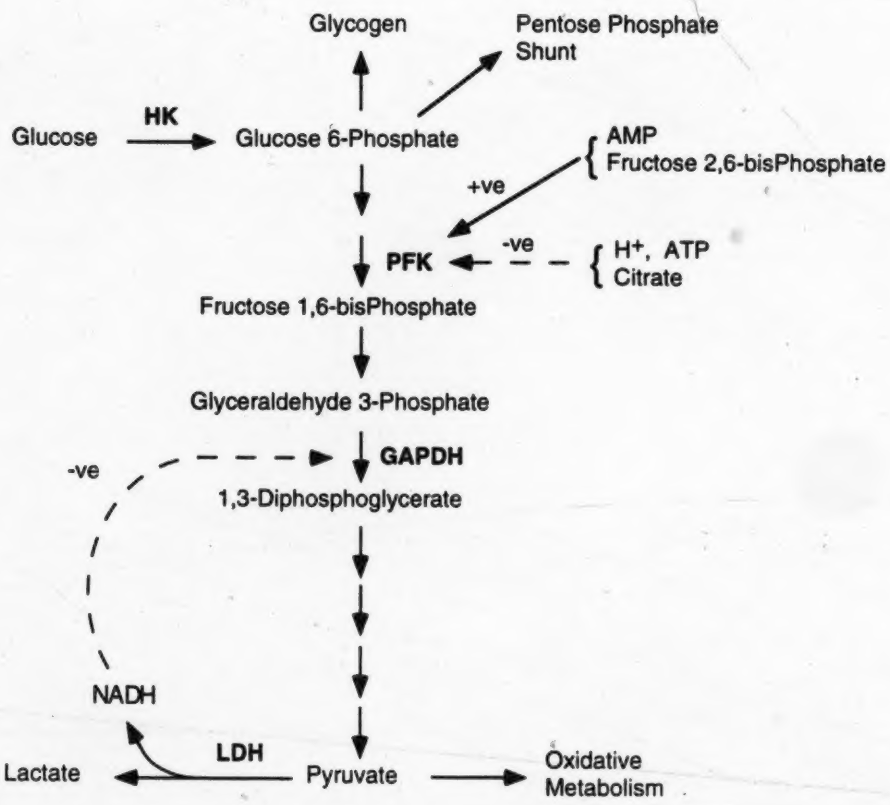

Figure I.1 Control points on the glycolytic pathway regulating glucose catabolism. PFK, phosphofructokinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase, LDH, lactate dehydrogenase, HK, hexokinase.

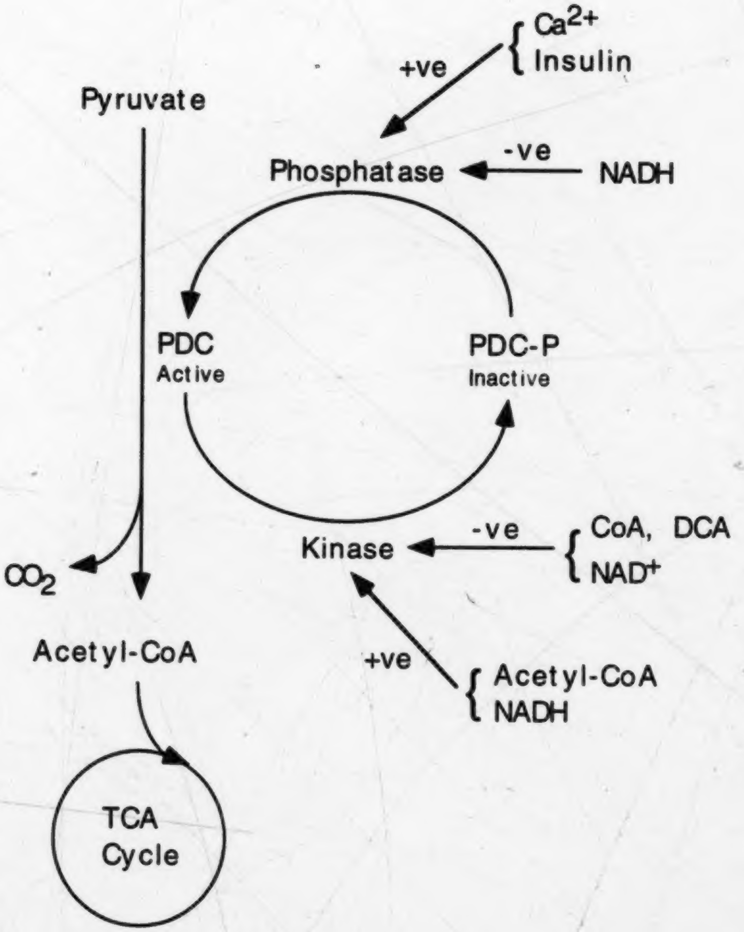

Figure I.2 Control of pyruvate dehydrogenase complex (PDC) activity. DCA, dichloroacetate.

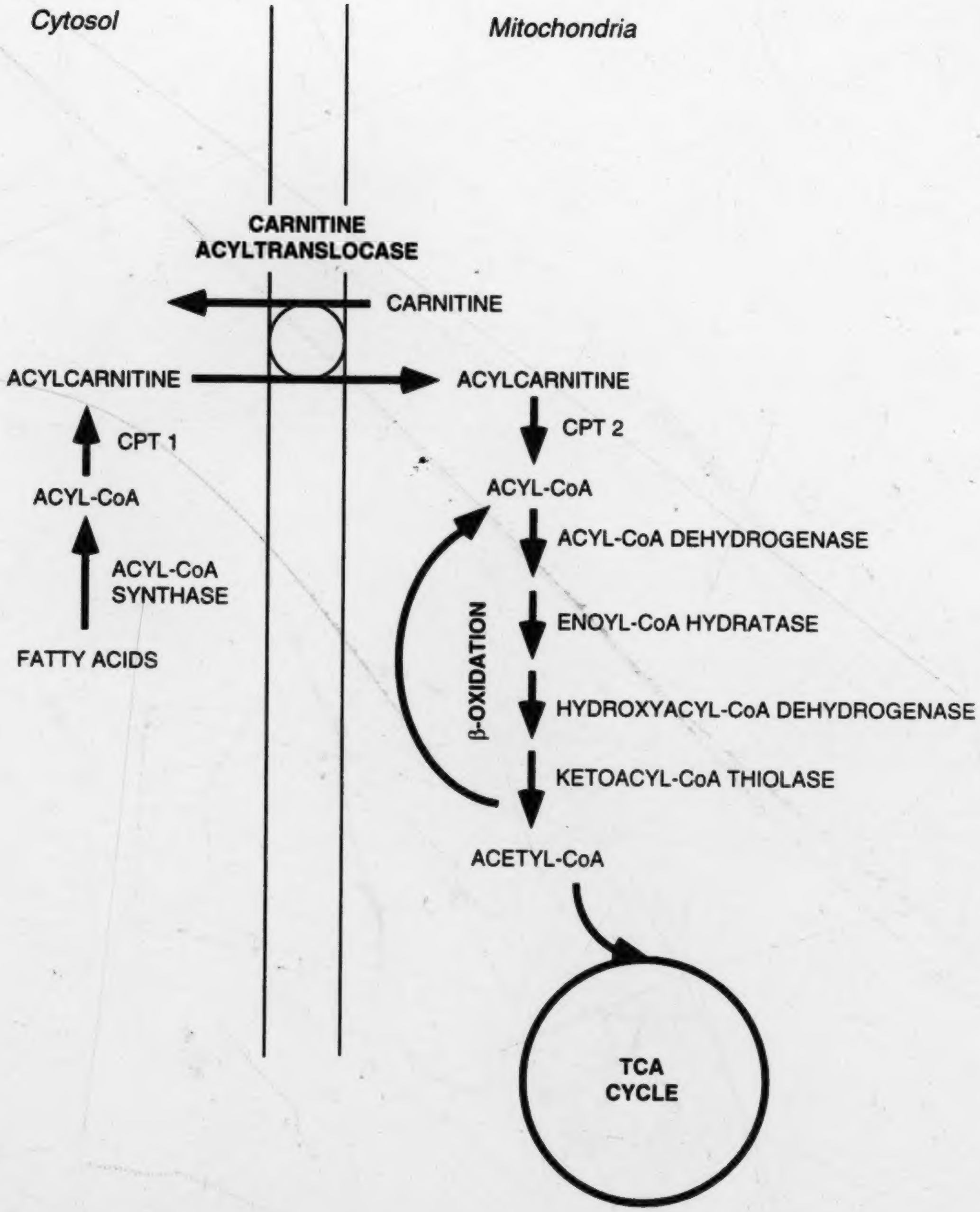

Figure I.3 β-oxidation of fatty acids.
CPT 1, carnitine palmitoyltransferase 1; CPT 2, carnitine palmitoyltransferase 2.

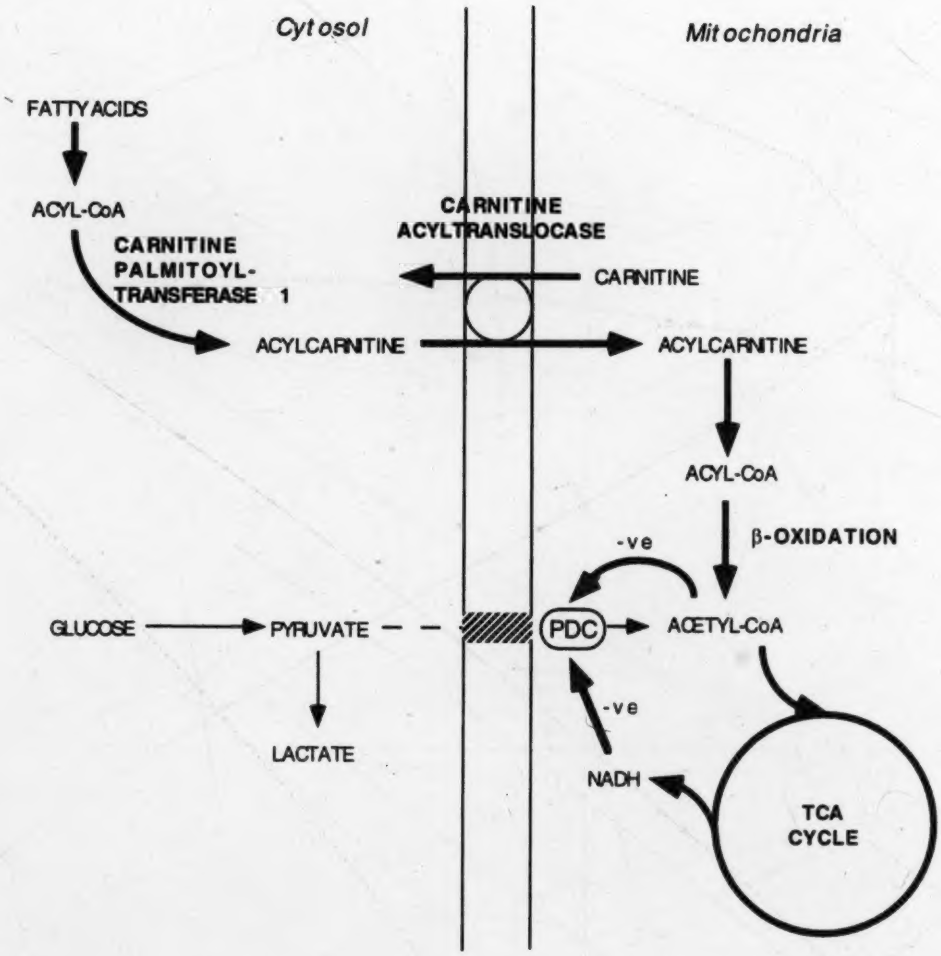

Figure I.4 Inhibition of pyruvate dehydrogenase complex by fatty acid oxidation.

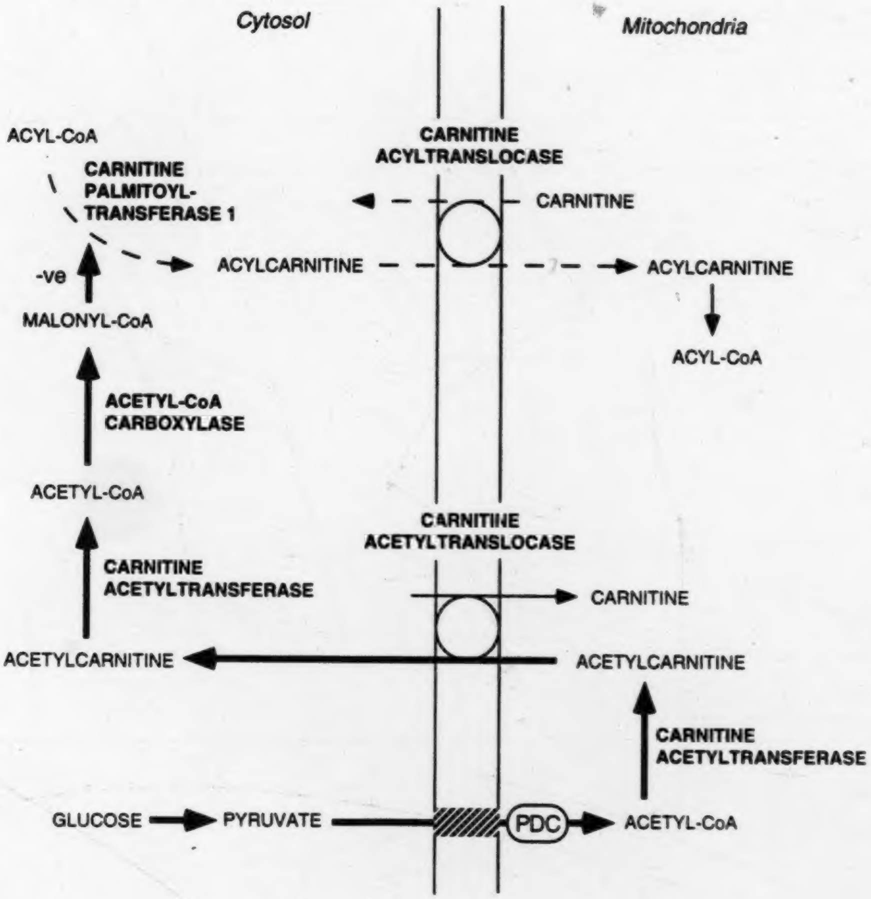

Figure I.5 Inhibition of fatty acid oxidation by glucose oxidation.

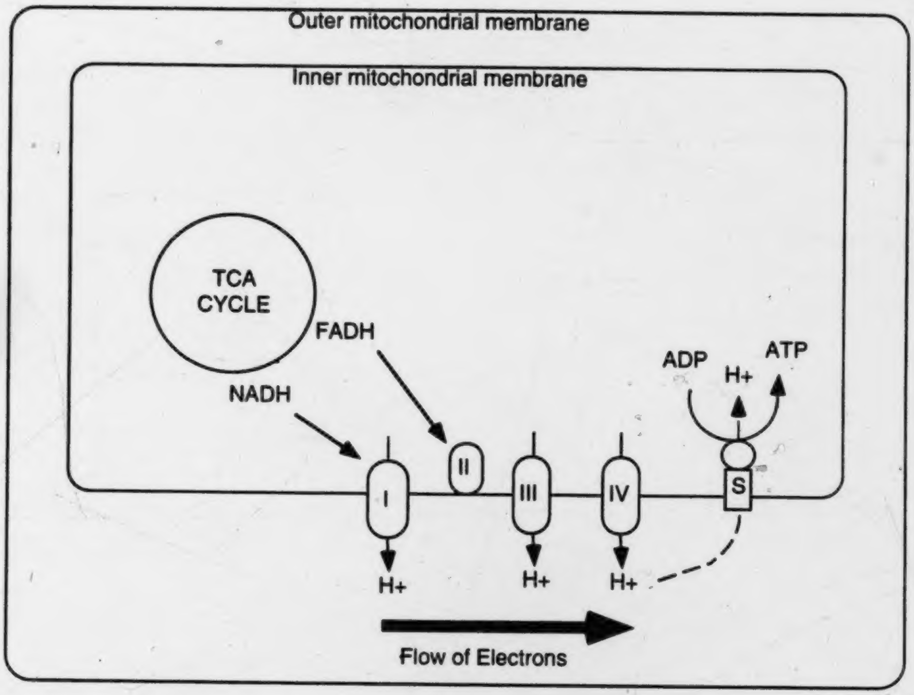

Figure I.6 Entry of electrons from NADH and FADH into the electron transport chain. S, ATP synthase. I; electron transport chain complex I; II, electron transport chain complex II; III, electron transport chain complex III; IV, electron transport chain complex IV. NADH feed electrons into complex I, while FADH feeds electrons in to complex II. As a result FADH can only lead to a maximal synthesis of 2 ATP, while NADH can lead to the synthesis of 3 ATP.

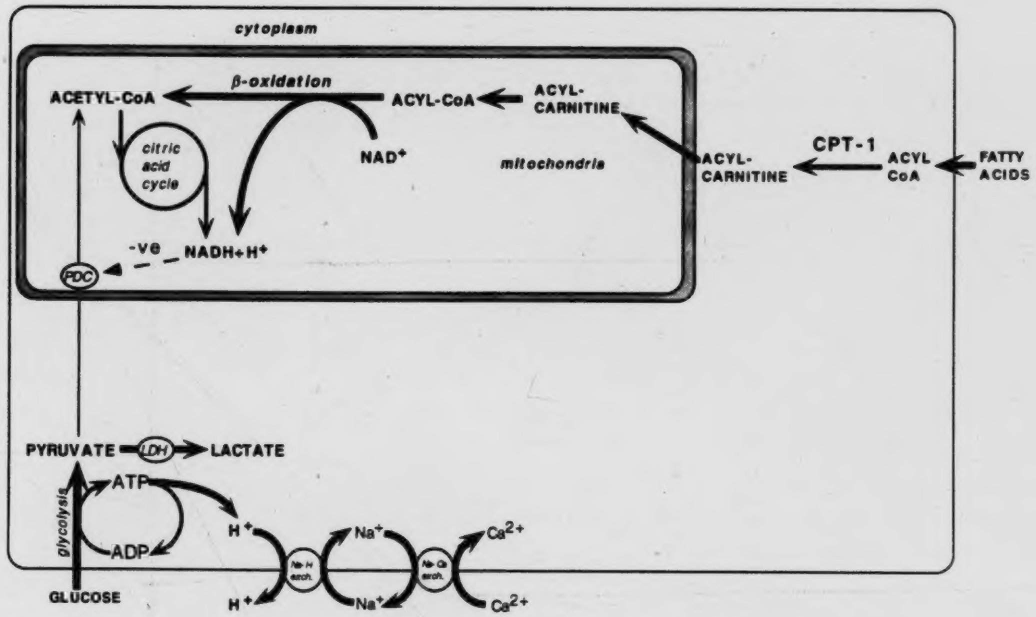

Figure I.7 H^+ production as a result of the dissociation between glycolysis and glucose oxidation and its contribution to Ca^{2+} overload. An increase in fatty acid oxidation leads to an inhibition of PDC activity and a reduction in glucose oxidation. As a result, pyruvate is converted to lactate, and the hydrolysis of ATP from glycolysis leads to increased H^+ production, which through the actions of the Na^+-H^+ and Na^+-Ca^{2+} exchangers leads to rise in intracellular Ca^{2+}. PDC, pyruvate dehydrogenase complex; CPT-1, carnitine palmitoyltransferase 1; Na-H exch. and Na-Ca exch.: sodium-hydrogen exchanger and sodium-calcium exchanger, respectively.

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Chapter II

Materials and Methods

1) Animals

Sprague-Dawley rats used as the cold sensitive species in this study were obtained from Charles River, Montreal, Canada. Rats were given rat chow and water *ad lib* and housed in shoe box cages at room temperature.

Richardson's ground squirrels (*Spermophilus richardsonii*) were live trapped near Edmonton, Canada. Following capture ground squirrels were kept in quarantine for at least 1 month. During the quarantine period, animal were housed individually in shoe-box cages and given rat chow (≈ 6% fat) and water *ad lib.* Following the quarantine period, the animals were either housed individually or placed in group cages with free access to a running wheel. No attempt was made to monitor or restrict animal access to the exercise wheel. Since ground squirrels exhibit a circannual transition between their hibernating and non-hibernating state (which can be determined by body weight) captured animals were maintained in the animal storage facilities for at least 2 months and weighed weekly to determine their state in the hibernation cycle. Those animals exhibiting a low and constant body weight were deemed to be in the non-hibernating phase, while those exhibiting weight gain followed by a high and constant body weight were deemed to be in the hibernating phase. Hibernation was induced by placing ground squirrels in a dark cold room at 4° C. No hibernating ground squirrel was used unless it underwent at least 2 bouts of hibernation.

All animals were cared for according to the guidelines set forth by the Canadian Council of Animal Care. All procedures performed on rats or ground squirrels received prior approval by either the Health Science Animal Welfare Committee, or the Biological Sciences Animal Welfare Committee.

2). Isolated Working Heart Model

2.1) Perfusion Solutions

Initial Langendorff perfusions used a Krebs'-Henseleit solution containing (in mM): NaCl (118.0), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (1.75 or 3.0), EDTA (0.5), $NaHCO_3$ (25.0) and glucose (11.0). Working heart perfusions used a modified Krebs'-Henseleit solution containing palmitate (1.2 mM) and 3% (w/v) bovine serum albumin (BSA, Fraction V, Boehringer). Palmitate was dissolved in 25 ml of an ethanol:water mixture (40:60%) containing 0.5-0.6 g Na_2CO_3/g of palmitate, while heating to evaporate the ethanol. Once the ethanol was evaporated the hot mixture was added to the 3% BSA Krebs'-Henseleit solution (without glucose) while stirring rapidly to ensure adequate binding of the palmitate to the BSA. The mixture is then dialyzed overnight in 5-6 volumes of normal Krebs'-Henseleit (without glucose) using 8000 MW cut-off SPECTRAPOR dialysis tubing (Spectrum Medical Industries, Los Angeles). Glucose (11 mM) was added to the working solution next day. The working solution was filtered through glass microfiber filters (GF/C, Whatman, Maidstone, England), and kept refrigerated at 5°C prior to use.

2.2) Working Heart Configuration

Male Sprague-Dawley rats or Richardson's ground squirrels (both sexes) were anesthetized with 60 mg/kg sodium pentobarbital. Heparin (150 units/kg) was then injected into the superior vena cava of ground squirrels 30 seconds prior to removal of the heart in order to prevent clot formation. Hearts were quickly excised and washed in ice cold Krebs'-Henseleit solution. Following this the aorta was cannulated, and a Langendorff perfusion with Krebs'-Henseleit solution (gassed with 95% O_2, 5% CO_2) at a pressure of 60 mmHg was initiated to wash out any remaining blood. During the Langendorff perfusion excess tissue was trimmed from the heart and the left atrium was cannulated to a preload reservoir set to deliver oxygenated working solution at a pressure of 11.5 mmHg. In hearts in which O_2 consumption was measured, the pulmonary artery was cannulated to a separate circuit containing an O_2 sensing electrode. If O_2 consumption was not being measured, the pulmonary artery was cut and solution was allowed to drain into the heart chamber resirvoir.

After a 10-15 minute period of Langendorff perfusion, hearts were switched to working mode by clamping off the aortic inflow line from the Langendorff reservoir while simultaneously opening the preload and afterload lines. Working heart solution was delivered to spontaneously beating hearts through the preload line (11.5 mmHg) and was ejected from the heart into a compliance chamber and onward in the afterload line which was preset to a column height of 80 mmHg (Figure I.1). Working heart solution was continuously pumped from the heart chamber resivour to an oxygenator via a peristaltic pump. Oxygenated solution was delivered to the left atrium via the preload line and pumped by the heart up the afterload line where it emptied back into the heart chamber reservoir. An injection port between the heart chamber reservoir and the oxygenator allowed for the addition of drugs and the withdrawal of solution samples.

Temperature control of hearts was maintained by warming or cooling the solution in the water jacketed heart chamber reservoir and oxygenator by two waterbaths preset to maintain heart temperature at 37° C or 15° C (5° C for the hibernating ground squirrel study). Cooling or heating of the heart was achieved by switching the circulation through the jacketed glassware from one water bath to the other. Hearts were allowed to beat spontaneously throughout the experimental protocol. Variations in afterload pressures were recorded by a Gould P21 pressure transducer and a Gould RS3600 physiograph (Gould Instruments, Cleveland, OH). These pressure recordings were used to obtain heart rate (HR) and peak systolic pressure (PSP) measurements. Cardiac output and aortic flow were measured using in-line flow probes (Transonic Systems, Ithaca, NY) attached to the preload and afterload lines, respectively. Coronary flow was determined by subtracting the difference between flow in the preload and after load lines. Cardiac power (CP) was calculated as the product of cardiac output and PSP.

2.3) Perfusion Protocol

Following the switch from the Langendorff to the working heart mode, hearts were perfused according to the protocol shown in Figure II.2. During the first normothermic period (labeled "control") hearts were perfused at 37° C for 30 minutes to establish pre-hypothermia baseline measurements for function and metabolism. During this time period, hearts which failed to maintain afterload flow, developed dysrhythmias or had a heart rate of less than 190 bpm were eliminated from the study (approx. 15%). Following this initial normothermic period hearts were cooled to 15° C over a 10 minute period. The 15° C hypothermia period was then maintained from minute 40 to minute 160 of the perfusion protocol (2 hours). At the end of 160 minutes, hearts were rewarmed from 15° to 37 ° C over a 10 minute period (rewarming period) following which the hearts were perfused for a further 30 minutes (termed "rewarmed"). This period constituted the time between minute 170 and minute 200 of the perfusion protocol.

Measurements of metabolism and mechanical function were obtained every 10 minutes during the normothermic (control, rewarmed) periods and every 30 minutes during the hypothermic period. At the end of the perfusion (200 minutes), hearts were frozen with Wollenberger clamps cooled to the temperature of liquid nitrogen. Depending on the experimental protocol, additional series of hearts were also frozen at the end of the control (30 minutes) and hypothermia (160 minutes) periods for analysis of glycogen, triacylglycerol and other metabolites.

3) Measurement of Energy Substrate Metabolism

Energy substrate metabolism was measured as described previously by Saddik and Lopaschuk (1991). Glycolysis and glucose oxidation were measured simultaneously in the working heart by perfusing with a solution containing [5-^{3}H/U-^{14}C]glucose (specific activity ≈ 200,000 dpm/ml of ^{3}H and ≈ 200,000 dpm/ml of ^{14}C). Palmitate oxidation was measured in a separate series of hearts, in which the solution contained [9,10-^{3}H] palmitate (specific activity ≈ 80,000 dpm/ml of ^{3}H). Solution samples for substrate utilization were collected every 10 minutes during the initial normothermic periods and every 30 minutes during hypothermia.

3.1) Measurement of Glycolysis

Glycolysis was measured by quantitatively collecting the $^{3}H_2O$ liberated from [5-^{3}H]glucose (Du Pont-New England Nuclear) at the triose phosphate isomerase and enolase steps of the glycolytic flux pathway (Figure II.3) (Hue and

Hers, 1974). The ^3H_2O produced as a result of glycolysis was separated from [^{3}H]glucose by passing 0.2 ml solution samples through columns containing 2 ml of a Dowex 1-X4 anion exchange resin (200-400 mesh) mixture. This technique results in a 98-99.6% separation of ^3H_2O from [^{3}H]glucose (Saddik and Lopaschuk, 1991). Dowex mixture was prepared by suspending dowex (90 g/L) in 0.4 M potassium tetraborate overnight. After loading into columns, the dowex was extensively washed with H_2O to remove potassium tetraborate prior to loading of solution samples. Columns were washed with 0.8 ml of H_2O to elute ^3H_2O and the sample was mixed with 10 ml of scintillation fluid (ECOLITE, ICN Radiochemicals, Irvine, CA) and subjected to standard liquid scintillation spectroscopy.

3.2) Measurement of Glucose Oxidation

Glucose oxidation was measured by using [U-^{14}C]glucose (Du Pont-New England Nuclear) and quantitatively measuring the rate of $^{14}CO_2$ produced by PDC and the TCA cycle (Figure II.4). This included $^{14}CO_2$ released as a gas and ^{14}C-bicarbonate retained in the solution. The $^{14}CO_2$ gas was collected by continuously bubbling the air outflow from the oxygenator into 50 ml of 1M hyamine hydroxide (methylbenzothonium in methanol, ICN Radiochemicals, Irvine, CA). Samples of hyamine hydroxide (0.3 ml) were placed directly into liquid scintillant for determination of ^{14}C content. Solution samples (3 ml) were withdrawn from the injection port at time intervals throughout the perfusion protocol (see Figure II.1), and stored under 2 ml of mineral oil to prevent liberation of $^{14}CO_2$ into the atmosphere. The ^{14}C contained in solution as bicarbonate was then liberated as $^{14}CO_2$ by mixing 1 ml of solution with 1 ml of 9 N H_2SO_4 in sealed reaction flasks. The liberated $^{14}CO_2$ was trapped in 300 µl of 1M hyamine hydroxide suspended in center-wells. The reaction flasks were gently agitated for 1 hour to completely liberate the $^{14}CO_2$ trapped in ^{14}C-bicarbonate. The hyamine samples and center-wells were then counted using ACS scintillation cocktail (ICN Radiochemicals) and standard scintillation counting techniques.

3.3) *Measurement of Palmitate Oxidation*

Palmitate oxidation was measured by quantitatively collecting the amount of ^3H_2O liberated from [9,10-^{3}H]palmitate (Du Pont-New England Nuclear) by the acyl-CoA dehydrogenase and L-3-hydroxyacyl-CoA dehydrogenase steps of the β-oxidation pathway and produced by the electron transport chain (Figure II.5). ^3H_2O was separated from [^{3}H]palmitate by extracting the hydrophobic [^{3}H]palmitate into chloroform (Saddik and Lopaschuk, 1991). Solution samples (0.5 ml) were treated with 1.88 ml of a chloroform:methanol mixture (1:2, v:v), followed by 0.625 ml of chloroform and 0.625 ml of a 2 M Kcl: 1.45 M HCl mixture. The aqueous phase was collected with a Pasteur pipette and treated with 1 ml of chloroform, 1 ml of methanol and 0.9 ml of the 2 M KCl: 1.45 M HCl mixture. Following separation of the different phases, 0.5 ml aliquots of the aqueous phase were combined with 5 ml of scintillation fluid (ECOLITE) and counted for ^{3}H using standard liquid scintillation techniques. This technique has previously been shown to result in a 99.7% separation of ^3H_2O from [^{3}H]palmitate (Saddik and Lopaschuk, 1991). Measurement of fatty acid oxidation was only performed in hearts where glycolytic flux was not being measured.

3.4) Measurement of O_2 Consumption.

O_2 consumption of isolated working hearts was determined by measuring the difference in the O_2 content of solution in the preload line and the effluent from the pulmonary artery. O_2 content of the solution was determined by using a single polarographic O_2 electrode (Yellow Spring Instruments, Boulder, CO) in a temperature controlled chamber. The O_2 electrode could be calibrated to 95% O_2 by passing the solution directly from the oxygenator over the surface of the electrode. By switching the flow going to the electrode from the oxygenator to the pulmonary artery effluent a direct determination of the percent O_2 consumption by the heart could be obtained using only a single O_2 electrode. This method readily allowed for re-calibration of the electrode when heart temperature was altered. Re-calibration of the electrode was required between temperatures as a change in temperature effects both electrode performance and the extent of O_2 saturation in solution. Myocardial O_2 consumption was calculated according to the following equation:

$$O_2 \text{ use} = \Delta\%O_2 \times CF \times \text{Atm. Press.} \div 760 \text{ Torr} \times O_2 \text{ Solubility} \div O_2 \text{ Density}$$

Where $\Delta\%O_2$ represents the difference between $\%O_2$ measured in the preload canula and $\%O_2$ measured in the pulmonary artery. CF represents coronary flow and Atm. Press. is the atmospheric pressure in mmHg. Of the factors used in the equation, both O_2 solubility and O_2 density are temperature dependent, with values for O_2 solubility and O_2 density in solution being 23.9 µl/ml (volume of gas per volume of liquid) and 0.03933 µmol/µl (moles per volume of gas) at 37° C and 34.0 µl/ml and 0.04233 µmol/µl at 15° C. To convert cardiac power/unit O_2 consumption to % cardiac efficiency, values for cardiac power and O_2 consumption were converted to units of Joules by using the conversion factors: and 1 mm Hg · ml = 1.33 x 10^{-4} J and 1 μmol O_2 = 0.4478 J, respectively (Suga, 1990).

4) Metabolite Measurements

4.1) Determination of Glycogen

Glycogen was extracted from hearts frozen at the end of the control, hypothermia and rewarmed periods according to the method of Bergmeyer and Grassl (1983). Powdered heart tissue (150-200 mg) was measured into test tubes and dissolved in 0.3 ml of 30% KOH (w/v) by immersion in boiling water for 1 hr. Glycogen was precipitated by the addition of 0.2 ml of 2% (w/v) Na_2SO_4 and 2.0 ml of absolute ethanol and stored overnight at -20° C. Following centrifugation at 3500 rpm for 5 minutes the supernatant was discarded and the precipitate washed once with 2.0 ml of 66% ethanol. Glycogen was converted to monosacharrides by boiling the precipitate in 1.0 ml of 3.79 M H_2SO_4 for 3 hr. The solution was neutralized with 10 M KOH in the presence of 0.33 M MOPS. Glucose concentration was determined by using a glucose determination kit (SIGMA, St. Louis, MO) which is based on the activity of glucose oxidase and peroxidase enzymes leading to the oxidation of dianisidine as the colorimetric reagent. After color development for 20 min at room temperature, the absorbance of each tube was measured at 475 nm using a spectrophotometer (DU-65, Beckman). Absorbance values were determined against a standard curve containing 0-20 μg of glucose. Glycogen content of heart tissue was expressed as μmol glucose/g dry weight.

4.2) Determination of Triacylglycerol

Triacylglycerol content of the heart was determined according to the method outlined by Lopaschuk et al (1986). Triacylglycerol content was determined by separating neutral and polar lipids, followed by determination of the glycerol content of the neutral lipid fraction. Tissue lipids were extracted and separated as described by Bowyer and King (1977). Frozen tissue (100 mg) was homogenized in 5 ml of ice cold chloroform:methanol for 40 seconds at full speed using a polytron homogenizer. After the addition of 0.75 ml of methanol, samples were vortexed and allowed to stand on ice for 1 hr before being centrifuged at 3500 rpm for 10 min. The resulting supernatant was completely evaporated under a stream of nitrogen at 50° C and resuspended in 2.5 ml of chloroform. Neutral lipids were separated from polar lipids by loading samples onto silicic acid columns and collecting the effluent. Columns were washed with 4 ml of chloroform to remove the remaining neutral lipids. The silicic acid columns were prepared by mixing 28.5 g of silicic acid (pre-dried overnight at 80° C) into 100 ml of chloroform and applying a 2.5 ml of this slurry to each column. A 0.5 ml aliquot of chloroform containing the neutral lipids was evaporated under a stream of nitrogen at 50° C and used in the determination of triacylglycerol content.

Triacylglycerol content in the sample was determined by using a Triglyceride G assay kit (Wako Pure Chemical Industries, Osaka). This assay involves hydrolyzing triacylglycerol into glycerol and fatty acids by using lipoprotein lipase. The glycerol is then incubated with glycerol kinase and glycerol 3-phosphate oxygenase to produce dihydroxyacetone phosphate and H_2O_2. Color development was measured by determining the extent of p-chlorophenol and 4-aminoantipyridine condensation at 505 nm as a result of H_2O_2 formation. Color formation was measured following the addition of 1 ml of enzyme reaction mixture to each tube containing the dried neutral lipid sample and incubating at 37° C for 15 minutes. Color formation was measured against a standard containing 0.0312% glycerol (w/v). Values for triacylglycerol were expressed as μmol·g dry^{-1} after conversion to molar values by using the molecular weight of Triolein (885 g·mol^{-1}).

4.3) Determination of Adenine Nucleotides and Creatine Phosphate

Adenine nucleotide (ATP, ADP, AMP) and creatine and creatine phosphate content of frozen hearts were determined via HPLC following extraction in 6% perchloric acid (PCA). Frozen heart tissue (100 mg) was ground in an ice cold mortar and pestle with 1 ml of 6% PCA and 0.5 mM EGTA. Following this samples were centrifuged for 10 min at 3500 rpm and 95 μl of dithiothreitol (0.32 M) was added to the resulting supernatant. The supernatant was then neutralized with 5 M K_2CO_3 and centrifuged at 3500 for 10 min to remove the potassium perchlorate precipitate. The resulting supernatant was then analyzed for high energy phosphate nucleotides and creatine and creatine phosphate according to the HPLC method of Ally and Park (1992).

4.4) Determination of Malonyl-CoA and Acetyl-CoA Content

Malonyl-CoA and acetyl-CoA levels in myocardial tissue were determined by HPLC following PCA extraction, as described by Saddik et al (1993). Frozen heart tissue (200 mg) was ground in an ice cold mortar and pestle with 1 ml of 6% PCA and 0.5 mM EGTA. Following this samples were centrifuged for 10 minutes at 3500 rpm and 95 μl of dithiothreitol (0.32 M) and 200 μl of MOPS was added to the resulting supernatant. Short chain acyl-CoAs were measured using HPLC.

5) Acetyl-CoA Carboxylase Analysis

5.1) Isolation of Acetyl-CoA Carboxylase

A cytosolic fraction containing acetyl-CoA carboxylase (ACC) was isolated using a polyethylene glycol (PEG) precipitation method (Kudo et al., 1995). All ACC isolation procedures were done at 4° C. Frozen heart tissue (200 mg) was homogenized in 0.4 ml of homogenization solution containing (in mM) tris base at pH 7.5 (50), mannitol (250), EDTA (1), EGTA (1), NaF (50), sodium pyrophosphate (5), phenylmethylsulfonyl floride (PMSF) (1), benzamidine (1), dithiothreitiol (1) and 4 μg/ml soy bean trypsin inhibitor. Following homogenization for 30 s using a PRO homogenizer at 1/2 speed, the sample was centrifuged at 14,000 x g for 20 min. The resulting supernatant was made to 2.5% PEG by adding a volume of 25% PEG 6000 stock solution (w/v) equivalent to 1/9 the volume of the supernatant. Samples were then vortexed for 10 min and centrifuged for 10 min at 10,000 x g. The resulting supernatant was then made to 6% PEG by the addition of 25% PEG stock to a volume of 3.5/19 of that of the supernatant. Samples were then vortexed for 10 min and centrifuged for 10 min at 10,000 x g. The resulting pellet was resuspended in a solution similar to the one used for the isolation except that mannitol was omitted, tris base concentration was 100 mM and glycerol (10% w/v) and NaN_3 (0.02% w/v) were added. Protein concentration was determined according to the bicinchoninic acid method (Smith et al., 1985). Protein concentration was diluted to 2.50 mg/ml for the ACC activity assay, or 2.25 mg/ml for separation of ACC isoforms using SDS-PAGE.

5.2) Measurement of Acetyl-CoA Carboxylase Activity

ACC activity was measured at 37 and 5° C by determining the incorporation of ^{14}C-bicarbonate into malonyl-CoA (Witters and Kemp, 1992). The assay solution consisted of (in mM): tris base (60.6) pH set to 7.5 at 37° and 5° C by the use of acetic acid, Na_2ATP (1.32), β-mercaptoethanol (2.12), Mg acetate (5.0), acetyl-CoA (1.06), $NaHCO_3$ (18.08) including 1.5 μCi of

$NaH^{14}CO_3$, 1 mg/ml BSA with or without citrate (10),. The reaction was started by the addition of 12.5 μg of protein (5 μl) to 160 μl of the reaction mixture. The incorporation of ^{14}C-bicarbonate into malonyl-CoA was stopped by the addition of 25 μl of 10% PCA after 1 min of incubation at 37 °C and 30 min of incubation at 5° C. Blank samples were obtained by the addition of PCA prior to the addition of enzyme to neutralize enzyme activity. Following centrifugation at 3500 rpm for 20 min 160 μl of the supernatant was transferred into glass minivials and dried under low heat to remove any remaining ^{14}C-bicarbonate. Once dried, the samples were resuspended in 100 μl of distilled water, mixed with 4 ml of scintillation cocktail (ECOLITE), and counted for residual ^{14}C.

5.3) Determination of Acetyl-CoA Carboxylase Isoforms

The ACC enzyme isoform content was analyzed by performing image analysis of a streptavidin blot. Protein samples for gel electrophoresis were prepared by diluting 50 μl of 2.25 mg/ml ACC protein with 25 μl of a solution containing 30 % glycerol, 3 % β-mercaptoethanol, 6 % SDS, 0.1 mg/ml bromophenol blue and 130 mM Tris (pH = 6.8). The resulting mixture was boiled for 5 minutes. Proteins were loaded onto a gel consisting of a 3 % acrylamide stacking gel and a 5 % acrylamide separating gel. Following separation, proteins were transferred onto nitrocellulose paper using a wet transfer apparatus and blocked overnight (≈ 16 hr) with 10% milk power in phosphate buffered saline (PBS). The nitrocellulose was then washed for 2 hr with PBS containing 1 % milk powder and peroxidase labeled streptavidin (1 μg/ml). The nitrocellulose was then washed 4 times with PBS containing 0.05 % Tween 20, and 3 times with PBS alone. Biotin containing proteins were visualized by washing the nitrocellulose with a chemiluminescent kit (Amersham) and exposing the nitrocellulose paper to x-ray film. The resulting blots were scanned into an image analysis program and the individual ACC isoforms (280 kDa, 265 kDa) were quantified.

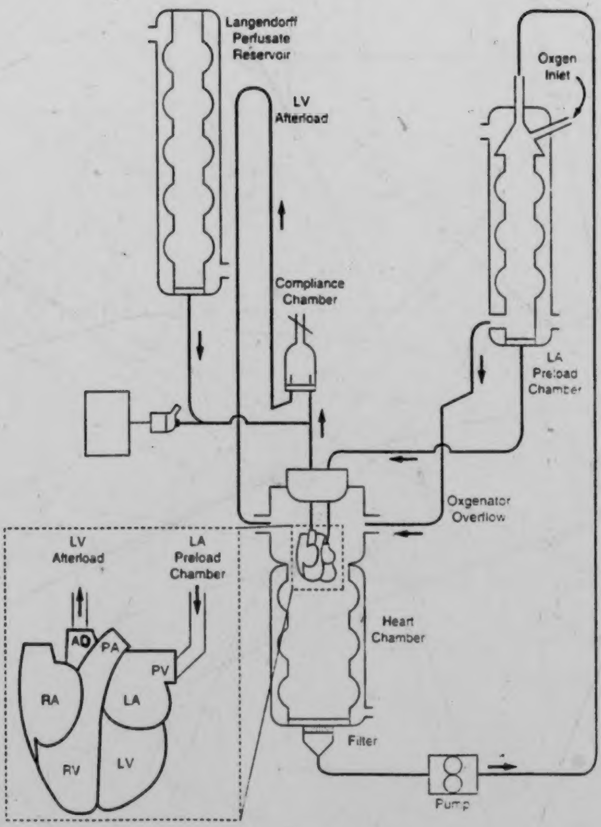

Figure II.1. Isolated working heart configuration. Working heart solution was pumped from the heart chamber into the left atrial preload chamber where it was oxygenated. From here the oxygenated buffer was delivered to the left atrium via the pulmonary vein (overflow from the left atrial preload chamber was returned to the heart chamber). Following filling of the left ventricle, the solution was ejected out the aorta and into the compliance chamber. From the compliance chamber the solution is forced up the afterload line and drains back into the heart chamber. PV, pulmonary vein; LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle; PA, pulmonary artery; AO, aorta. (Figure adapted from Barr and Lopaschuk, 1996)

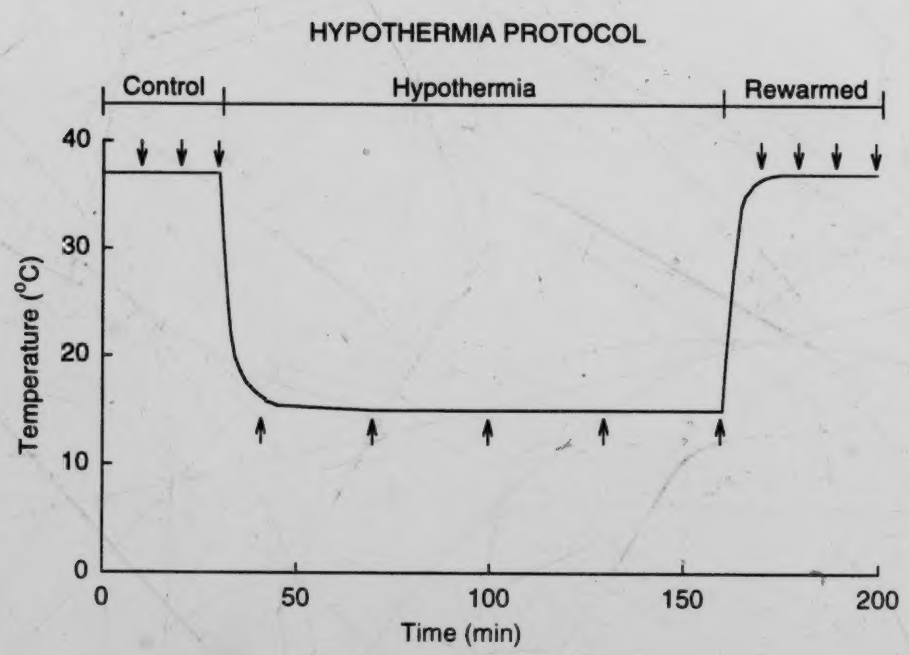

Figure II.2 Perfusion protocol for isolated working ground squirrel and rat hearts. Arrows indicate the time points at which functional parameters were assessed and buffer samples for substrate metabolism were taken.

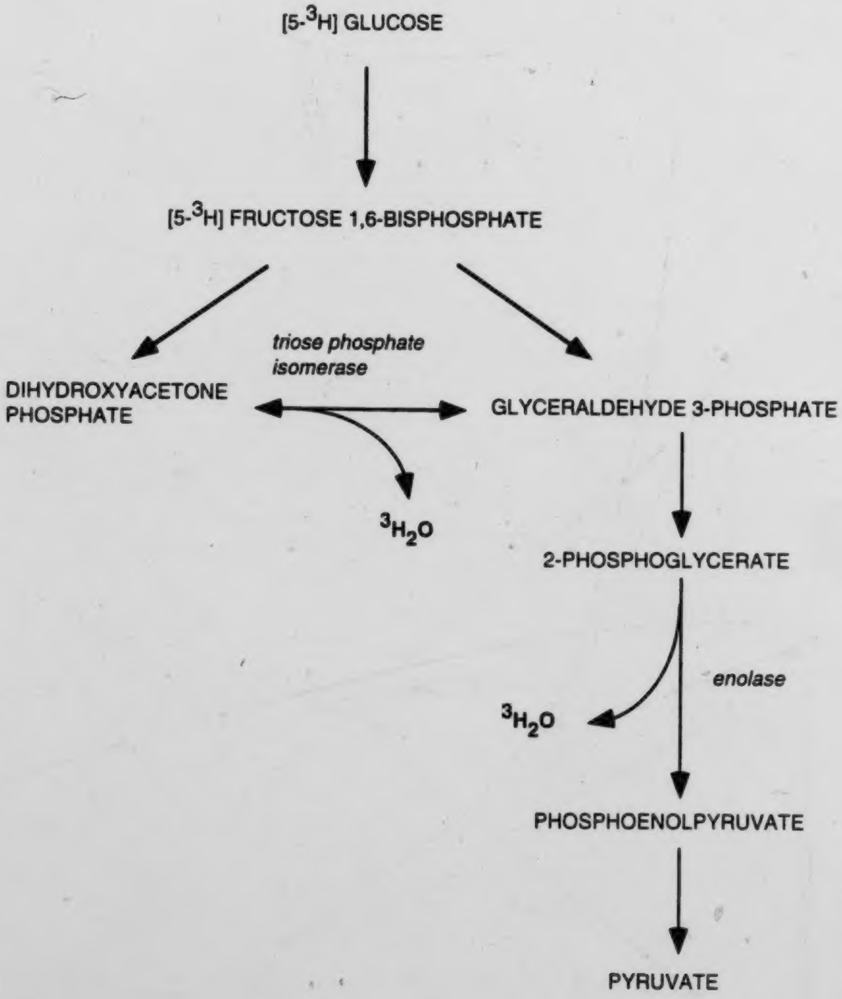
[5-3H] GLUCOSE
[5-3H] FRUCTOSE 1,6-BISPHOSPHATE
triose phosphate isomerase
DIHYDROXYACETONE PHOSPHATE
GLYCERALDEHYDE 3-PHOSPHATE
3H2O
2-PHOSPHOGLYCERATE
enolase
3H2O
PHOSPHOENOLPYRUVATE
PYRUVATE

Figure II.3 Liberation of ^3H_2O from [5-^{3}H]glucose as a measure of glycolytic rate. ^3H_2O was created at either the triose phosphate isomerase or enolase step in the glycolytic pathway and measured as an index of glycolytic rate.

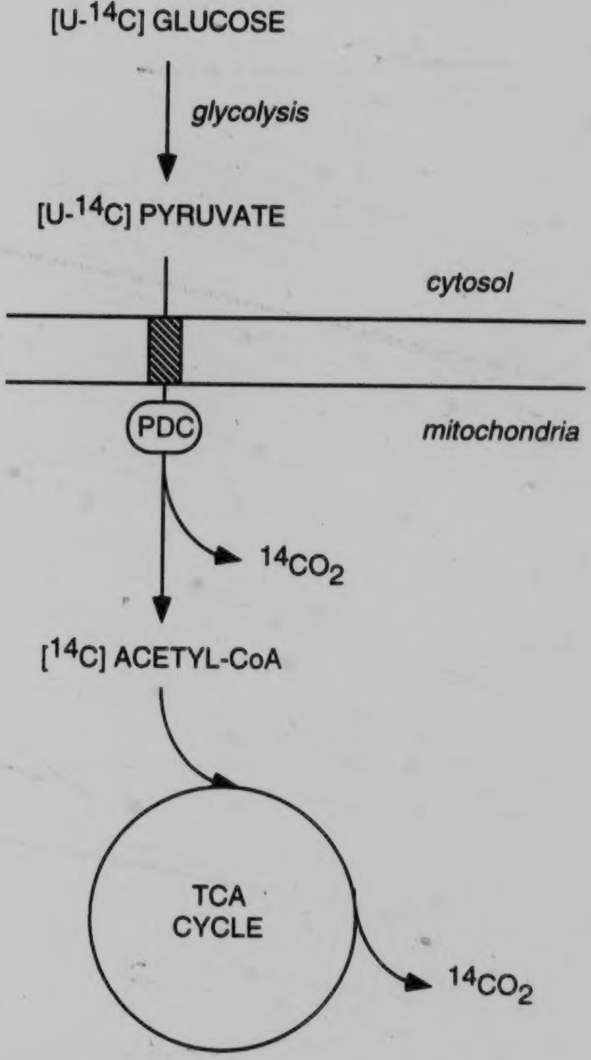

Figure II.4 Liberation of $^{14}CO_2$ from [U-^{14}C]glucose as a measure of glucose oxidation. $^{14}CO_2$ liberated by the pyruvate dehydrogenase complex and the TCA cycle served as an index of glucose oxidation rate.

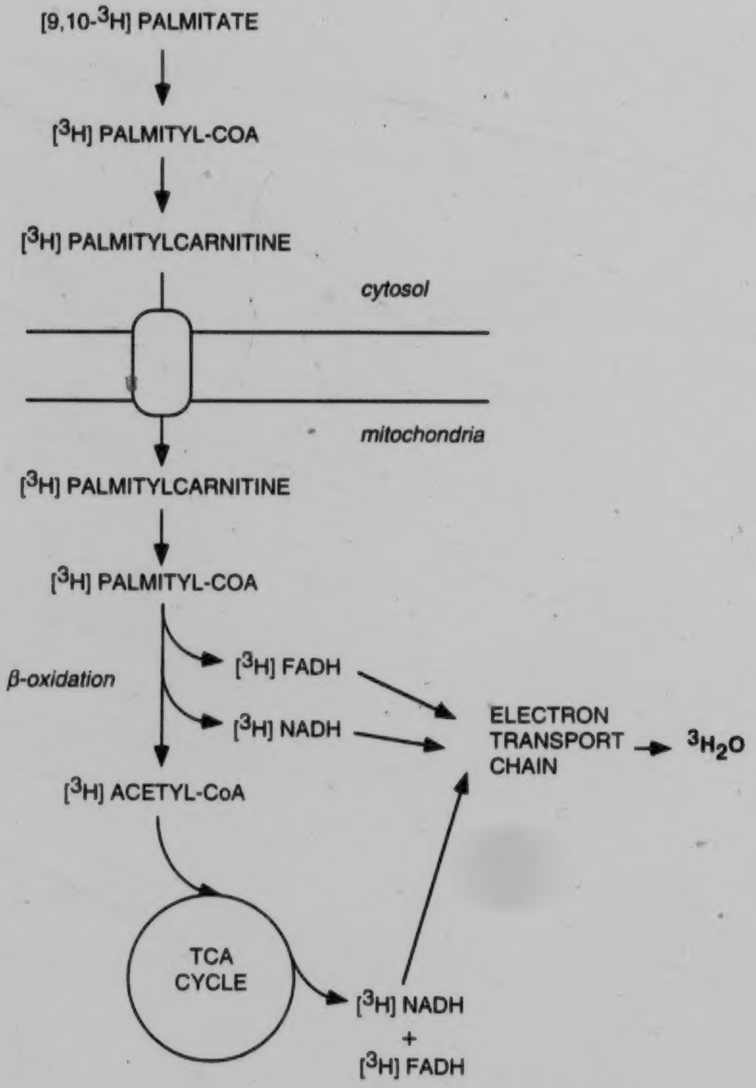

Figure II.5 Liberation of ^3H_2O from [9,10-^{3}H]palmitate as a measure of palmitate oxidation. ^3H_2O was created by the electron transport chain from [^{3}H]-NADH and [^{3}H]-FADH from β-oxidation and the TCA cycle.

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Chapter III

The Effects of Fatty Acids on Glucose Metabolism During Hypothermia and Rewarming in Rat Hearts

Introduction

Energy substrate metabolism plays an important role in determining the recovery of heart function following an episode of ischemia. Exposing a heart to a high concentration of fatty acids during reperfusion following ischemia can depress the recovery of function (Lopaschuk et al., 1994, Fralix et al., 1992). The detrimental effect of fatty acids has been linked to a suppression of glucose oxidation. Stimulation of glucose oxidation either directly or indirectly results in an improvement in functional recovery (McVeigh and Lopaschuk, 1990; Lopaschuk et al., 1988; Lewandowski and White, 1995; Broderick et al., 1993).

In hypothermic hearts, recent studies by Mjos et al (1991) and Steigen et al (1994) have shown that, as with ischemia-reperfusion, exposing hypothermic hearts to high concentrations of fatty acids leads to a poor recovery of function upon rewarming. Despite the widespread use of hypothermia for heart preservation during surgery, little is known about the direct effects of hypothermia on glucose metabolism and how fatty acids affect glucose metabolism during hypothermia and following rewarming. Animal studies have implicated a hypothermia-induced depression in whole body glucose metabolism on the basis of increased plasma glucose levels and the decreased oxidation of radiolabelled [^{14}C]glucose (Hoo-paris et al., 1988; Jourdan, 1991; Tveita et al., 1996). Whether similar changes occur directly in the heart have not been clearly established. Analysis of arterio-venous differences in substrate concentrations across the heart has suggested that glucose metabolism is depressed during hypothermia (Shida et al., 1977, Russ and Lee, 1965). Although these studies suggest that hypothermia alters glucose metabolism in the heart, the inability to control the substrate composition of the plasma in hypothermic animals may effect the interpretation of these results. Changes in the concentration of non-glucose substrates such as pyruvate, lactate, ketone bodies and fatty acids under hypothermic conditions can alter glucose metabolism, making attempts to draw any conclusions about the effects of hypothermia on glucose metabolism difficult.

To determine directly the effects of hypothermia on glucose metabolism, isolated working hearts were perfused with radiolabelled glucose (^{3}H, ^{14}C) in the presence and absence of 1.2 mM palmitate. The effects of fatty acids on glucose metabolism and heart function during hypothermia and rewarming were also studied. This study tested the hypothesis that high concentrations of fatty acid retain the ability to depress glucose metabolism regardless of heart temperature, and that fatty acid inhibition of glucose metabolism depresses the functional recovery of rewarmed hearts.

Methods

Animals

Sprague-Dawley rats were used in this study.

Heart Perfusions

Isolated working heart perfusions were performed as outlined in Chapter II. The hypothermia-rewarming perfusion protocol was also as described in Chapter II.

Perfusion Conditions

The Krebs'-Henseleit solution used in the experiment contained 1.25 mM free Ca^{2+} with or without 1.2 mM palmitate. Working heart solution without palmitate was made according to the method outlined in Chapter II except that palmitate and sodium carbonate were omitted. Hearts were perfused according to the hypothermia protocol outlined in Chapter II.

Measurement of Glycolysis, Glucose Oxidation and Palmitate Oxidation

As outlined in Chapter II, glycolysis and glucose oxidation were measured simultaneously in the working hearts by perfusing them with [5-^{3}H/U-^{14}C]glucose.

Statistical Analysis

Single Factor Analysis of Variance with a Student-Neuman-Keuls test was used to determine if values were significantly different between the control, hypothermia and rewarmed periods within a group, while an unpaired t-test was used when making comparisons between hearts perfused with or without palmitate. A value of $p < 0.05$ was considered to be significant. All values presented represent the mean ± S.E.M.

Results

The Effects of 1.2 mM Palmitate on Function

The effect of adding 1.2 mM palmitate to the perfusion medium on the functional parameters averaged over the normothermic and hypothermic periods is shown in Tables III.1 and III.2. Heart rate, peak systolic pressure and stroke volume are shown in Table III.1, while coronary flow, cardiac output and cardiac power are shown in Table III.2. All parameters were significantly reduced as a result of cooling the heart to 15° C. The reduction in these parameters could be mainly attributed to the large reduction in heart rate, with the resulting reduction in cardiac output affecting coronary flow and cardiac power, both of which are dependent on cardiac output. The decrease in heart rate was accompanied by an increase in stroke volume so that the decrease in cardiac output was not as great as the decrease in heart rate. The addition of 1.2 mM palmitate to the perfusion solution led to a significant reduction in cardiac power during the initial normothermic control period compared to hearts perfused with glucose alone. The addition of palmitate also significantly decreased coronary flow, peak systolic pressure, cardiac output and cardiac power during the hypothermic period. Following rewarming to 37° C, peak systolic pressure, cardiac output and cardiac power were depressed in both groups compared to the pre-hypothermic control period values. However, along with coronary flow, these parameters were all depressed to a greater extent in the hearts perfused with palmitate, as compared to the hearts perfused with glucose alone. With the exception of heart rate, many of these parameters were not stable during the rewarmed period, and show a continual decay over time. This effect is demonstrated in Figure III.1 which shows the change in cardiac power as a function of time over the course of the experiment. Cardiac power was stable over the control period, and although depressed as a result of cooling, it showed no sign of decay over the 2 hour hypothermic period. Following hypothermia, the highest value for cardiac power for either group was obtained immediately following rewarming (170 min), after which a continuous decay in cardiac power occurred during the remainder of the rewarmed period. As palmitate depressed cardiac power during the initial normothermic control period, values for cardiac power during rewarming were also plotted as a percentage of their average pre-hypothermia (control period) values (Figure III,2). Even when the results obtained during rewarming were normalized for pre-hypothermia differences, palmitate led to a greater suppression of cardiac function than hearts perfused with glucose alone. Immediately following rewarming (170 min), cardiac power recovered to 77% of control period values in the glucose alone group, but only 43% in the glucose and palmitate group.

The Effects of 1.2 mM Palmitate on Glucose Metabolism

The effect of 1.2 mM palmitate on steady state glycolytic flux during normothermic and hypothermic perfusion is shown in Figure III.3. Hypothermia decreased glycolytic rates in both groups. Glycolytic rates decreased approximately 6 fold upon cooling in hearts perfused with glucose alone and approximately 10 fold in hearts perfused with palmitate. Upon rewarming, glycolytic rates returned to pre-hypothermia control period values. The addition of palmitate to the perfusion medium led to a significant decrease in glycolytic rates over the course of the perfusion protocol. During the initial control period, the addition of palmitate depressed glycolytic rates to 25% of the values obtained using glucose alone. During hypothermia and following rewarming, the glycolytic rates in hearts perfused with palmitate are depressed to approximately 15 and 22%, respectively, during these perfusion periods, in comparison with hearts perfused with glucose alone.

The effect of palmitate on steady state glucose oxidation is shown in Figure III.4. The pattern of hypothermia and rewarming on glucose oxidation rates was similar to that observed for glycolysis, although glucose oxidation was not depressed to the same extent by hypothermia as glycolysis. Cooling hearts to 15° C led to an approximate 3 fold decrease in glucose oxidation in both groups. During rewarming, the rate of glucose oxidation recovered to, or surpassed, the pre-hypothermic control value for each group. One of the major differences between glycolytic and glucose oxidation rates for the two groups, was the magnitude by which palmitate depressed these two parameters. During the control period, palmitate depressed glucose oxidation rate to approximately 10% of the value observed in the hearts perfused with glucose alone. The ratio of glycolysis to glucose oxidation for the different periods is shown in Table III.3. The results indicate that palmitate led to a greater uncoupling of glycolysis from glucose oxidation over the course of the experiment. Hypothermia, in general, leads to a greater coupling between glycolysis and glucose oxidation.

As a result, both palmitate and hypothermia are capable of altering glucose metabolism independent of the actions of each other. Furthermore, glycolysis and glucose oxidation are not affected equally by this treatment, with glycolysis being affected to a greater extent by hypothermia, and glucose oxidation being inhibited to a greater extent by palmitate.

Table III.1 The effects of 1.2 mM palmitate on heart rate, peak systolic pressure and stroke volume during normothermic and hypothermic perfusion of isolated working rat hearts.

	Heart Rate *(beats·min^{-1})*	PSP *(mm Hg)*	Stroke Volume *(ml·beat^{-1})*
Control			
Glucose	224 ± 10	132 ± 5	0.24 ± 0.02
Glucose + Palmitate	228 ± 7	121 ± 5	0.19 ± 0.01
Hypothermia			
Glucose	33 ± 2*	101 ± 3*	0.37 ± 0.05*
Glucose + Palmitate	27 ± 3*	71 ± 4*†	0.30 ± 0.06*
Rewarmed			
Glucose	220 ± 7	108 ± 2	0.16 ± 0.02*
Glucose + Palmitate	246 ± 13	99 ± 3	0.10 ± 0.01*

Values are the mean ± S.E.M. for 6 hearts perfused with 11 mM glucose and 7 hearts perfused with 11mM glucose and 1.2 mM palmitate. PSP, peak systolic pressure.

*, significantly different from respective control values.

†, significantly different from hearts perfused without palmitate.

Table III.2 The effects of palmitate on coronary flow, cardiac output and cardiac power during normothermic and hypothermic perfusion of isolated working rat hearts.

	Coronary Flow $(ml \cdot min^{-1})$	Cardiac Output $(ml \cdot min^{-1})$	Cardiac Power $(mm\ Hg \cdot ml \cdot min^{-1} \cdot 10^{-2})$
Control			
Glucose	25.3 ± 2.3	55.2 ± 6.3	67 ± 6
Glucose + Palmitate	22.6 ± 2.2	44.3 ± 2.9	51 ± 6†
Hypothermia			
Glucose	11.7 ± 1.5*	12.1 ± 1.5*	10 ± 1*
Glucose + Palmitate	6.2 ± 1.3*†	6.6 ± 1.0*†	5 ± 1*†
Rewarmed			
Glucose	22.9 ± 1.8	36.3 ± 5.1*	36 ± 4*
Glucose + Palmitate	16.1 ± 3.5*	16.6 ± 3.4*†	17 ± 4*†

Values are the mean ± S.E.M. for 6 hearts perfused with 11 mM glucose and 7 hearts perfused with 11mM glucose and 1.2 mM palmitate.

*, significantly different from respective control values.

†, significantly different from hearts perfused without palmitate.

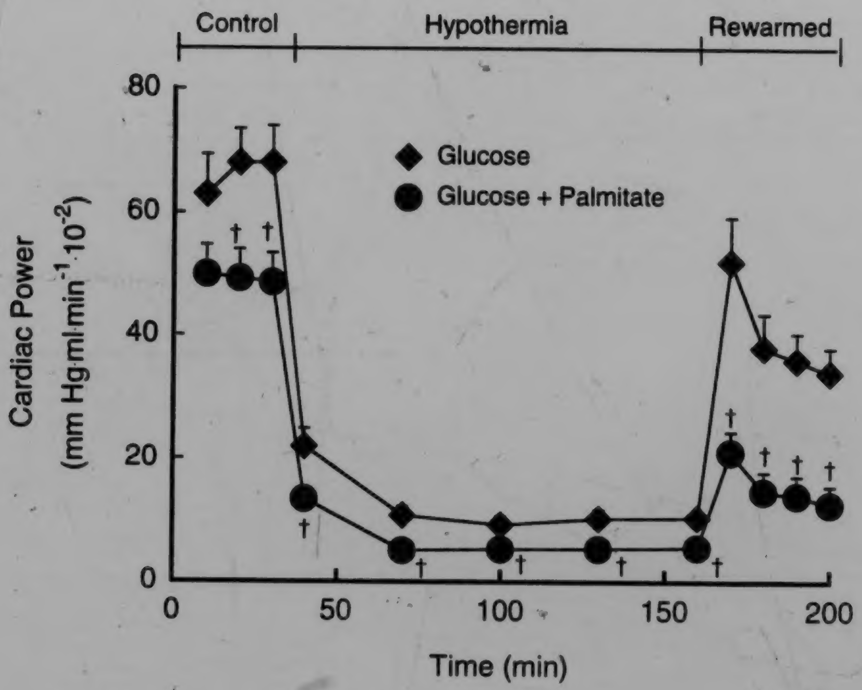

Figure III.1 Changes in cardiac power over the course of the perfusion protocol. Values represent mean ± S.E.M. for 6 hearts perfused with 11 mM glucose and 7 rat hearts perfused with 11 mM glucose and 1.2 mM palmitate.

†, denotes values significantly different from hearts perfused without palmitate.

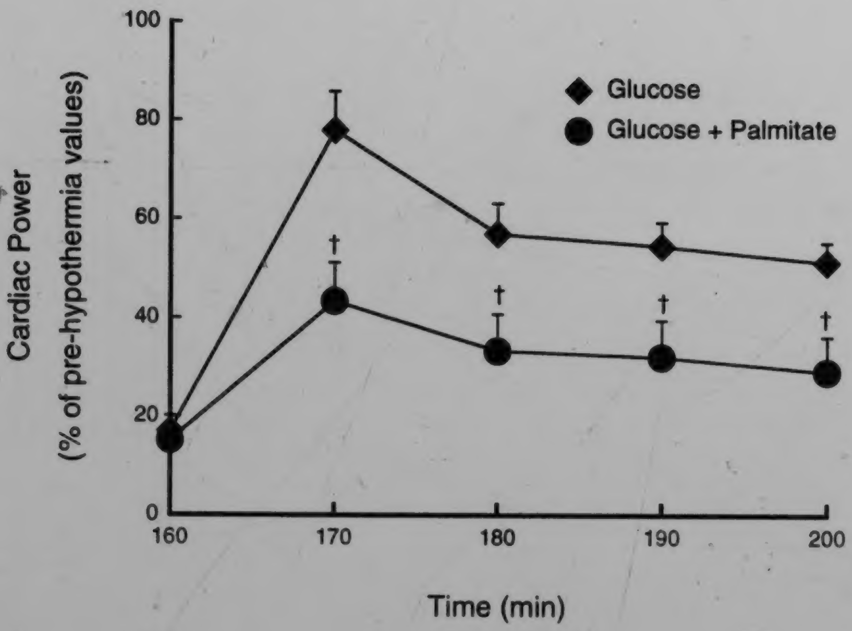

Figure III.2 The effect of rewarming on cardiac power normalized as a percentage of pre-hypothermia control period values. Values represent mean ± S.E.M. for 6 hearts perfused with 11 mM glucose and 7 hearts perfused with 11 mM glucose and 1.2 mM palmitate.

†, denotes values significantly different from hearts perfused without palmitate.

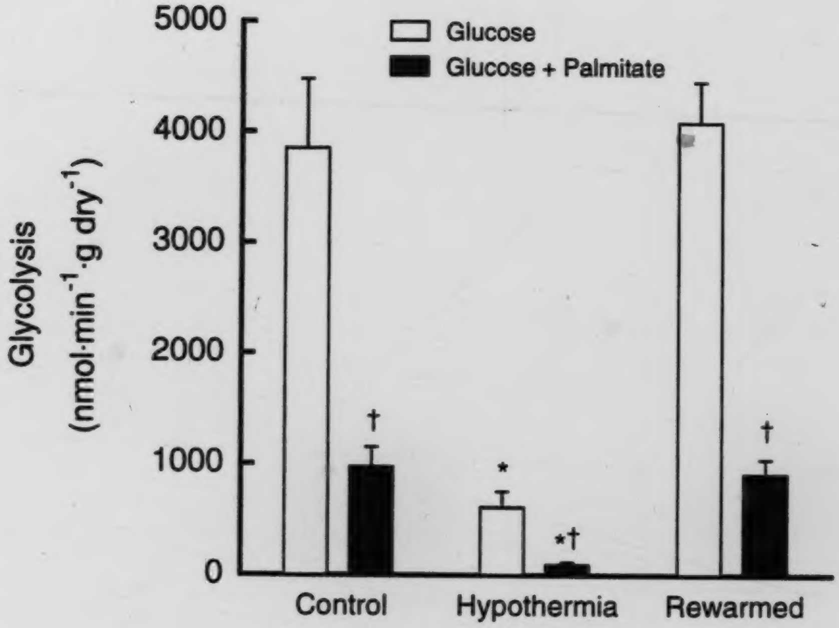

Figure III.3 The effect of palmitate on steady state rates of glycolysis in hypothermic and rewarmed hearts. Values represent mean ± S.E.M. for 6 hearts perfused with 11 mM glucose and 7 hearts perfused with 11 mM glucose and 1.2 mM palmitate.

*, significantly different from respective control values.

†, significantly different hearts perfused without palmitate.

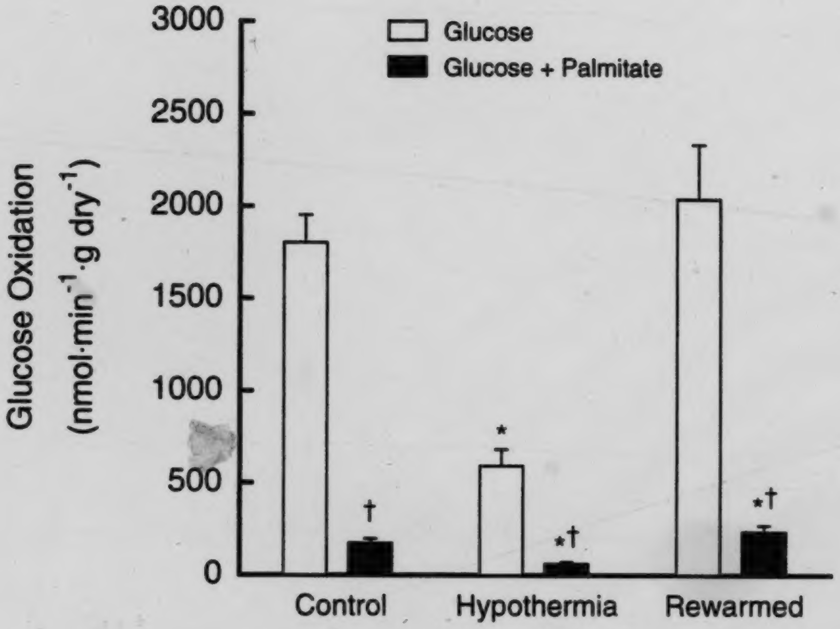

Figure III.4 The effect of palmitate on steady state rates of glucose oxidation in hypothermic and rewarmed hearts. Values represent mean ± S.E.M. for 6 hearts perfused with 11 mM glucose and 7 hearts perfused with 11 mM glucose and 1.2 mM palmitate.

*, significantly different from respective control values.

†, significantly different from hearts perfused without palmitate.

Table III.3 The effect of palmitate on the ratio of glycolysis to glucose oxidation in hypothermic and rewarmed hearts.

	Glycolysis / Glucose Oxidation
Control	
Glucose	2.1
Glucose + Palmitate	5.6
Hypothermia	
Glucose	1.0
Glucose + Palmitate	1.6
Rewarmed	
Glucose	2.0
Glucose + Palmitate	3.9

Discussion

This study examined the effects of both fatty acids and hypothermia on glucose metabolism and heart function. We observed a greater depression in cardiac power output following rewarming in hearts exposed to high levels of fatty acids. These results support the observation by Mjos et al (1991) and Steigen et al (1994) that fatty acids can suppress the recovery of function in hearts rewarmed following hypothermia. We have expanded on these previous studies to show that fatty acids are capable of depressing glucose metabolism during and following hypothermia. This effect is similar to the reduction in function observed in hearts during following ischemia-reperfusion with high levels of fatty acids. As observed during hypothermia-rewarming in the present study, fatty acids during ischemia-reperfusion can also lead to a dramatic uncoupling of glycolysis from glucose oxidation (Lopaschuk et al., 1993), suggesting that a similar mechanism may be affecting both conditions.

Although coronary flow was depressed during hypothermia in the present study, it is unlikely that the hearts were ischemic, as glycolysis was depressed to a greater extent than glucose oxidation, the opposite of what would be expected for these two parameters as a result of ischemia. Similarly, the greatest depression of glycolysis coincided with the lowest coronary flow rate during hypothermia in the palmitate perfused hearts, again suggesting that these hearts were not ischemic. The depression in cardiac power during hypothermia was also greater than the depression in coronary flow, again suggesting that substrate and O_2 supply were adequate for the level of function. Together, these observations suggest that the results observed in the present study were solely due to the effects of hypothermia-rewarming, and not due to the development of ischemia within the hearts during the hypothermic perfusion.

In this study, glycolysis and glucose oxidation were measured to determine if alterations in glucose metabolism could explain the detrimental effects of fatty acids on hypothermic hearts. Hypothermia resulted in a general depression of both glycolysis and glucose oxidation relative to normothermic control period values. This effect may have occurred as the result of either a direct inhibition of enzymes involved in glucose metabolism, or indirectly through a reduction in mechanical function and a subsequent reduction in metabolic demand. The reduction in glycolysis by 6 and 10 fold in hearts perfused with glucose alone and those perfused with palmitate, respectively, roughly matches the reduction in cardiac power observed in these groups upon cooling. While this observation suggests that glycolytic rate is controlled by the demand placed on the heart as a result of mechanical function, it should be noted that glycolytic rates did recover to pre-hypothermia control period levels following rewarming despite a poor recovery of cardiac power in both groups. As a result, our data suggests that glycolysis is not strictly governed by the level of mechanical function in these hearts over the course of the perfusion protocol.

In contrast to glycolysis, glucose oxidation was only depressed by 2-3 fold as a result of hypothermia, suggesting that PDC activity is also not strictly regulated by changes in mechanical function. As with glycolysis, this observation was also supported by the complete recovery of glucose oxidation following rewarming despite a reduction in mechanical function. Under normothermic and hypothermic conditions, glucose oxidation rate is regulated both by metabolic demand and the relative availability of substrates from both carbohydrate and non-carbohydrate sources. Metabolic demand is based on ATP utilization by both work related and non-work related processes, both of which may be independently affected by hypothermia and by rewarming. Thus while metabolic demand and temperature play a role in regulating glucose metabolism in the heart, they do not act simply through changes in the level of cardiac power output being performed by the heart. Conversely, the recovery of glucose metabolism upon rewarming suggests that no permanent damage to the glycolytic pathway or glucose oxidation occurs as a result of cooling and rewarming which might account for the differences in heart function. As a result, it appears that the changes in glucose metabolism observed as a result of hypothermia and rewarming cannot be ascribed solely to either heart temperature or metabolic demand in the form of cardiac power output.

From the results obtained in this study, it appears that the decrease in glucose metabolism observed in whole animals subjected to hypothermia (Hooparis et al., 1991; Shida et al., 1977) is likely due to the greater reduction in glycolysis than glucose oxidation. Similarly, the differences in the effect of hypothermia on glycolysis and glucose oxidation may explain why lactate utilization is not depressed to the same extent as glucose utilization in either whole animals or heart tissue subjected to hypothermia (Jourdan 1991; Shida et al., 1977; Teoh et al., 1988), as lactate bypasses glycolysis and is oxidized by the mitochondria following conversion to pyruvate.

In addition to the general effects of hypothermia in depressing glucose metabolism, the addition of palmitate to the perfusion medium led to a depression in both glycolysis and glucose oxidation under normothermic and hypothermic conditions. The depression of glycolysis and glucose oxidation by palmitate under normothermic conditions is similar to previous observations (Schonekess et al., 1995). The depression of glucose oxidation by fatty acids was greater than fatty acid inhibition of glycolysis, resulting in a higher ratio of glycolysis to glucose oxidation (Table III.3). Even under hypothermic conditions where glucose metabolism was already depressed, fatty acids were able to depress glucose oxidation to a greater extent than glycolysis, suggesting that even during hypothermia the oxidation of palmitate is capable of exerting a greater inhibitory effect on PDC and glucose oxidation than on PFK and the glycolytic pathway. The fact that palmitate depressed glycolytic flux during hypothermia despite the depression already brought about by cooling suggests that regulatory enzymes, such as PFK, retain their sensitivity to regulation by fatty acid oxidation under these conditions. Thus the effect of fatty acids in depressing the recovery of mechanical function following hypothermia and rewarming may result from the ability of fatty acids to depress glucose metabolism under both normothermic and hypothermic conditions.

In summary, we were able to show that the addition of 1.2 mM palmitate to the perfusion medium led to a depression in the recovery of function following rewarming from hypothermia. The addition of 1.2 mM palmitate to the perfusion medium led to a depression in both glycolysis and glucose oxidation; however, glucose oxidation was depressed to a greater extent than glycolysis. This effect was observed under both hypothermic and normothermic conditions, indicating that fatty acids retain the ability to alter glucose metabolism during hypothermia. In addition to the effects of 1.2 mM palmitate on glucose metabolism, hypothermia itself altered glucose metabolism so that glycolytic rate was depressed to a greater extent than the rate of glucose oxidation. Hypothermia did not permanently affect glucose metabolism as both glycolytic and glucose oxidation rates recovered following rewarming from hypothermia.

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Chapter IV

Comparison of Metabolism and Function Between the Rat and Richardson's Ground Squirrel Heart During and Following Hypothermia

Introduction

Since the introduction of hypothermia as a protective element in surgery, researchers have commented on the superior ability of hibernating animals to withstand the damaging effects of low temperature (Bigalow et al., 1950; Hearse et al., 1981), and have suggested that these adaptations would be beneficial to improving the survival of the myocardium during hypothermic storage. Early studies comparing myocardial physiology in hibernating and non-hibernating species focused on differences in metabolism and function at low temperatures. Zimny and Taylor (1965) examined electro-cardiographic and metabolite changes in the hearts of rats and ground squirrel subjected to whole body hypothermia. They found an increased accumulation of lactate and disruption of the mitochondria was associated with whole body hypothermia in rats but not ground squirrels. However, as they failed to control for the possibility of hypoxia as a result of depressed respiration, these results do not accurately reflect the effects of hypothermia alone. In isolated Langendorff perfused rat and ground squirrel hearts, hypothermia leads to a decreased energy charge (ATP/ADP +P_i) in rat hearts but not in ground squirrel hearts (Burlington et al., 1976). The authors interpret this as indicating a better balance between energy synthesis and utilization in ground squirrels than rats. Caprette and Senturia (1984) examined the effects of hypothermia on the isovolumetric performance of isolated rat and ground squirrel hearts perfused in Langendorff mode. They observed that the ground squirrels were able to maintain a better pressure development at low temperatures than rats, and were more responsive to electrical stimulation than rats. Burlington and Darvish (1988) observed a similar effect in the isolated working heart model perfused with a modified Krebs'-Henseleit solution containing 1.2 mM palmitate. Under these conditions pressure development and cardiac output are maintained in ground squirrel hearts but not rat hearts at low temperatures. Burlington's group (Burlington et al, 1989) also found that ground squirrel hearts were able to regulate coronary flow in relation to changes in perfusion pressure at low temperatures while rat hearts could not below 17° C. The authors interpret these results as indicating that the ground squirrel retain the capacity to regulate myocardial perfusion at low temperatures in response to changes in demand, while the rat does not retain the capacity or have the reserve to regulate myocardial perfusion. Despite this loss in capacity, however, the rat hearts did not exhibit any signs of metabolic distress. Together, these results suggest that myocardial physiology differs between cold-sensitive and cold-tolerant species at low temperatures.

Although studies suggest that hypothermia affects both functional mechanics and energy metabolism differently in the hearts of cold-sensitive and cold-tolerant species, heart function and energy substrate metabolism have never been compared in these species during either hypothermia or rewarming. While Mjos et al (1991) and Steigen et al (1994), have shown that perfusion of rat hearts with 1.2 mM palmitate depresses coronary flow during hypothermia, and is detrimental to the recovery of function following rewarming, Burlington and Darvish (1988) have shown that ground squirrel hearts retain a level of function during hypothermia, superior to the rat, even in the presence of 1.2 mM palmitate. This effect may be related to the fact that during hibernation these animals must be adapted to utilizing fatty acids as a source of energy (South and House, 1967;

Galster and Morrison, 1966; Suomalainen and Sarikoski, 1967), and adapted not only to surviving low temperatures, but also to varying temperatures (0 - 37° C), as these animals must rewarm periodically (Lyman and Blinks, 1959; Lyman, 1965). That cold-tolerance appears to be an innate property of hearts from hibernating species regardless of season and physiological state within the annual hibernation cycle (Burlington and Darvish, 1988; Caprette and Senturia, 1984), suggests that these animals retain this adaptation throughout the calendar year.

The purpose of this study was to examine energy substrate metabolism and cardiac function in both rats (cold-sensitive species) and Richardson's ground squirrels (cold-tolerant species) during hypothermia and rewarming in the presence of high levels of fatty acids, to determine if functional recovery following rewarming differs between these species. This study also examines energy substrate metabolism to determine if species differences in substrate metabolism might account for differences in the recovery of function following rewarming.

Methods

Animals

The animals used in this study were Sprague-Dawley rats (cold-sensitive species) and Richardson's ground squirrels (*Spermophilus richardsonii*) (cold-tolerant species) which were trapped near Edmonton, Canada. Ground squirrels used in this study were all in the non-hibernating phase of their annual hibernation cycle.

Heart Perfusions

Isolated working heart perfusions were performed as outlined in Chapter II. The hypothermia-rewarming perfusion protocol was also as described in Chapter II.

Perfusion Conditions

The Krebs'-Henseleit solution used in the experiment contained 1.25 mM free Ca^{2+}. Hearts from both species were perfused according to the hypothermia protocol outlined in Chapter II. Hearts were frozen at the end of the control, hypothermic and rewarmed periods for analysis of high energy phosphate metabolites and endogenous substrate (glycogen and triacylglycerol) levels. An additional series of hearts were perfused at 37 and 15° C to examine O_2 consumption at these temperatures.

Measurement of Glycolysis, Glucose Oxidation and Palmitate Oxidation

As outlined in Chapter II, glycolysis and glucose oxidation were measured simultaneously in the working hearts by perfusing them with [5-^{3}H/U-^{14}C]glucose. Palmitate oxidation was measured in a separate series of hearts perfused with [9,10-^{3}H] palmitate. The contribution of glucose and palmitate to supplying acetyl-CoA to the TCA cycle was calculated by using the stoichiometric ratio of 2 mol acetyl-CoA/mol glucose and 8 mol acetyl-CoA/mol palmitate. Hydrogen ion (H^+) production from the imbalance between glycolysis and glucose oxidation was calculated for each heart by subtracting the rate of glucose oxidation from glycolysis and multiplying the resulting number by two as outlined by Lopaschuk et al (1993).

Determination of High Energy Phosphates.

ATP, ADP, AMP and creatine and creatine phosphate levels were determined by HPLC following PCA extraction as outlined in Chapter II.

Determination of Glycogen and Triacylglycerol Levels

Glycogen and triacylglycerol levels were determined as outlined in Chapter II.

Statistical Analysis

Single Factor Analysis of Variance with a Student-Neuman-Keuls test was used to determine if values were significantly different between control, hypothermia and rewarmed periods within a group, while an unpaired t-test was used when making comparisons between ground squirrels and rats. A paired t-test was used to compare values obtained at 37° C to those obtained at 15° C when O_2 measurements were made at these temperatures. A value of $p < 0.05$ was considered to be significant. All values represent the mean ± S.E.M.

RESULTS

Mechanical Function

Control Period

Changes in heart rate, peak systolic pressure and cardiac power averaged for the various periods of the perfusion protocol are shown in Table IV.1, while values for coronary flow, cardiac output and the ratio of coronary flow to cardiac power are shown in Table IV.2. Major species differences during the initial normothermic (control) perfusion was confined to coronary flow, which was significantly lower in rat hearts. Despite this difference in coronary flow, cardiac power output did not differ significantly between the two species, and cardiac power was stable over the course of the control perfusion period (Figure IV.1).

Hypothermia Period

Cooling the hearts to 15° C resulted in a significant reduction in all functional parameters measured in both species (Tables IV.1 and IV.2). Major species differences were observed in coronary flow, cardiac output and cardiac power. Although reduced, cardiac power output was maintained at a constant level over the course of the 2 hr hypothermic perfusion in both species (Figure IV.1), suggesting that hypothermia *per se* does not lead to a decay in cardiac performance over time. The level of cardiac power output obtained from rat hearts during hypothermia was significantly lower than that obtained from ground squirrel hearts despite the similar values of cardiac power observed in both species during the initial control period. This suggests that some aspect of myocardial physiology, acutely sensitive to cooling, leads to an immediate reduction of cardiac performance in cold sensitive species. Although hypothermia resulted in a significant reduction in coronary flow in both species (with coronary flow lower in rats than ground squirrels) it is unlikely that the delivery of O_2 and substrates was limiting cardiac power output, as values for coronary flow normalized for cardiac power actually increase as a result of hypothermia (Table IV.2).

Rewarmed Period

Following rewarming to 37° C, the functional parameters return to control period values in ground squirrel hearts but remain depressed in rat hearts. Although heart rate fully recovers for rat hearts, peak systolic pressure, cardiac output and coronary flow are all significantly depressed; as a result, cardiac power output obtained from these hearts is depressed (Figure IV.1). Although cardiac power is maintained at a constant level in ground squirrel hearts, it continues to decay in rat hearts throughout the rewarmed period. Although coronary flow is depressed in rats following rewarming, there is no difference in the level of coronary flow normalized for cardiac power between rats and ground squirrels (Table IV.2), suggesting that a reduction in coronary flow is unlikely to be the main cause of the poor recovery of cardiac performance in rat hearts.

Energy Substrate Metabolism

Glycolysis

The effects of hypothermia-rewarming on glycolysis over the course of the experiment is shown in Figure IV.2. The level of glycolytic flux measured at the individual time points was lower in ground squirrel hearts than in rat hearts over the course of the experiment. The time course demonstrates that glycolysis was linear over the normothermic (control, rewarmed) and hypothermic perfusion periods. Glycolysis was also linear following rewarming, however, a slight increase in glycolysis in rat hearts by the end of the experiment (200 min) suggests that the depression in cardiac function by this time point may have resulted in hearts which were mildly ischemic. However, glycolytic rate remained constant over the course of the rewarmed period despite the constant decay in cardiac function. The average steady state rate of glycolysis for the normothermic and hypothermic periods is shown in Table IV.3. The steady state rate of glycolysis tended to be higher in rat hearts than in ground squirrel hearts (over the course of the experiment), however, glycolytic rate did not different significantly between species. Hypothermic perfusion led to a significant decrease in glycolytic rate in both species relative to control period values. Glycolysis was depressed 15 fold in ground squirrel hearts and 8 fold in rat hearts. Following rewarming glycolytic, rates recovered to pre-hypothermic values and tended to be slightly elevated in both species, regardless of species differences in the recovery of cardiac function.

Glucose Oxidation

The effect of hypothermia-rewarming on glucose oxidation over the course of the experiment is shown in Figure IV.3. The time course demonstrates that the rate of glucose oxidation was linear during the normothermic and hypothermic perfusion periods; however, unlike glycolysis, the transition between normothermia and hypothermia did not significantly affect glucose oxidation. This effect is observed for the steady state rate for glucose oxidation measured in the two species (Table IV.3). The steady state rate of glucose oxidation was similar between the two species during the initial normothermic control period. Under hypothermic conditions the rate of glucose oxidation decreased to approximately 50% of control period values in ground squirrel hearts and remained unaffected by hypothermia in rat hearts. Following rewarming the rate of glucose oxidation increased in both species and was significantly higher than the rate observed during the initial control period (Table IV.3).

Palmitate Oxidation

Palmitate oxidation measured over the course of the experiment is shown in Figure IV.4. The level of palmitate oxidation at each time point was significantly higher in rat than ground squirrel hearts over the course of the experiment. Palmitate oxidation was linear during the normothermic and hypothermic periods. Palmitate oxidation decreased slightly in rat hearts at the end of the experimental protocol (200 min), suggesting that the depressed level of cardiac function may have been limiting the oxidation of palmitate at this time point. The average steady state rate of palmitate oxidation during the normothermic and hypothermic perfusions is shown in Table IV.3. The rate of palmitate oxidation was significantly higher in rat hearts than ground squirrel hearts during the initial control period, and remained significantly higher during the hypothermic perfusion. Unlike the results obtained for glucose oxidation, hypothermia resulted in a significant decrease in the rate of palmitate oxidation in both species relative to control period values. Palmitate oxidation decreases to approximately 1/5 of control in both species as a result of hypothermia. Following rewarming, the rate of palmitate oxidation recovered to pre-hypothermia control period levels in ground squirrel and rat hearts.

Metabolism Normalized for Cardiac Work

As changes in energy substrate metabolism during the hypothermia and rewarming periods may result from the direct effect of temperature on enzyme activity, as well as changes in cardiac power output (i.e. metabolic demand); substrate metabolism for the individual pathways was also normalized for cardiac power (Table IV.4). Under these conditions, the depression in glycolysis observed during hypothermia was not significant, suggesting that glycolytic flux changed in parallel with the changes in cardiac power during hypothermia.

In contrast, changes in oxidative metabolism in response to the level of cardiac power output differed greatly between the species. Glucose oxidation was significantly increased in both species during hypothermic perfusion and following rewarming. Palmitate oxidation differed between the species in response to hypothermia. Although no difference in palmitate oxidation normalized for cardiac work is observed between the species during the initial control period, hypothermia results in a slight increase in rat hearts, but a significant decrease in ground squirrel hearts. Following rewarming, palmitate oxidation returns to control period levels in ground squirrel hearts but is significantly elevated in rat hearts. These results suggest that energy utilization is altered in rat hearts during hypothermia and rewarming so that energy substrate metabolism is no longer directed toward cardiac power output and normal cardiac function. Ground squirrel hearts retain this coupling between energy metabolism and cardiac power output. The increased contribution of glucose oxidation in ground squirrel hearts during hypothermia is accompanied by a decrease in the contribution of palmitate oxidation to cardiac output.

Glucose Metabolism During Rewarming

Although cardiac power is depressed in rat and ground squirrel hearts during hypothermia it is stable over this time period. The greatest species difference in cardiac power output occurs following rewarming, suggesting that rewarming itself may affect heart function. During rewarming (160-170 min.) both heart temperature and functional parameters are increasing continuously (non-linearly), making any determination of a complete metabolic profile difficult.

However, as glycolysis and glucose oxidation are measured in the same hearts, these parameters will be simultaneously affected by the changes in temperature and function which occur constantly over this time period. The effects of rewarming on the rates of glycolysis and glucose oxidation for the two species are shown in Table IV.5. Although the rate of glucose oxidation during rewarming was similar between the species, the rate of glucose passing through glycolysis is lower in ground squirrel than rat hearts. This suggests that glycolysis is affected differently as a result of rewarming between the two species.

Hydrogen Ion Production

H^+ production as a result of the imbalance between glycolysis and glucose oxidation over the course of the experiment is shown in Figure IV.5, with steady state rates being shown in Table IV.6. In both species H^+ production is blunted as a result of hypothermia but recovers following rewarming. During hypothermia the rate of H^+ production decreases to a fraction of that observed during normothermic perfusion (Table IV.6). This is due to the fact that during hypothermia glycolysis is depressed to a greater extent than glucose oxidation and the two rates become more tightly coupled. No significant species differences in the rate of H^+ production is noted during either the normothermic (control, rewarmed) or hypothermic perfusion periods; however, H^+ production tends to be higher in rats than ground squirrels during these periods. During the period of rewarming (160-170 min), H^+ production in ground squirrels was significantly lower than that observed in rats due to the slower recovery of glycolysis during rewarming in ground squirrels (Table IV.5). Although the rate of H^+ during this period is not steady state due to changes in heart temperature as well as function, the average rate of H^+ production in ground squirrels (646±191 nmol·min^{-1}·g dry-

^{1}) was significantly less than that observed in rats (2625±561 nmol·min^{-1}·g dry^{-1}).

Contribution of Glucose and Palmitate to TCA Acetyl-CoA

The contribution of glucose and palmitate to acetyl-CoA for TCA cycle oxidation in ground squirrel and rat hearts are shown in Table IV.7. In both species, palmitate supplies the bulk (≈95%) of acetyl-CoA for the TCA cycle during the initial control period. As palmitate oxidation is significantly higher in rat hearts than ground squirrel hearts during this time period, total acetyl-CoA production from glucose and palmitate is also significantly higher in the rat. During hypothermia, the total acetyl-CoA obtained from substrate metabolism decreases significantly in both groups; however, the level obtained from rat hearts remain significantly higher than that obtained from ground squirrel hearts. As glucose oxidation is less inhibited by the decrease in temperature than palmitate oxidation, the contribution of glucose to total acetyl-CoA production increases at the expense of palmitate in both species increasing from < 5% to > 10% during hypothermia. Following rewarming, total acetyl-CoA production recovers to pre-hypothermia levels for both groups.

Cardiac Efficiency During and Following Hypothermia From Substrate Metabolism

The efficiency of translating energy substrate metabolism into cardiac power was determined by dividing the level of cardiac power by the rate of ATP production, which was calculated from substrate metabolism assuming efficient conversion of acetyl-CoA to ATP in the mitochondria. The effect of hypothermia and rewarming on cardiac efficiency is shown in Figure IV.6. During the control period, cardiac efficiency did not differ between rat and ground squirrel hearts.

However, during hypothermia cardiac power was significantly higher in the ground squirrel hearts when compared to rat hearts (Table IV.1), despite the fact that overall substrate metabolism was significantly lower (Table IV.7). As a result, the efficiency of translating ATP production into cardiac power increased significantly in ground squirrel hearts during hypothermia, and decreased slightly in rat hearts (Figure IV.6). This suggests that hypothermia results in an increase in non-work related energy expenditure in rat hearts and a reduction in ground squirrel hearts. Following rewarming cardiac efficiency returned to pre-hypothermic control period values in ground squirrel hearts, but was significantly depressed in rat hearts. The poor cardiac efficiency in rat hearts was primarily due to a poor recovery of cardiac power despite a nearly complete recovery of substrate metabolism to pre-hypothermic levels.

Cardiac Efficiency From O_2 Consumption

To determine if the changes in cardiac efficiency calculated from the level of energy substrate metabolism observed during the control and hypothermia periods accurately reflect changes in the relationship between cardiac function and substrate metabolism; cardiac efficiency was determined from a series of hearts perfused at 37 and 15° C using O_2 consumption as an index of metabolic change (Table IV.8). Under these conditions cardiac power and O_2 consumption decreased in both species as a result of hypothermia. The change in cardiac efficiency observed under these conditions matched that calculated from substrate metabolism, with cardiac efficiency increasing in ground squirrel hearts and decreasing in rat hearts upon cooling. Cardiac efficiency (when expressed as % efficiency) decreased in rat hearts from 13.8 ± 1.4% to 9.5 ± 0.9% upon cooling and increased in ground squirrel hearts from 11.8 ± 2.2% to 17.0 ± 3.0%. This suggests that the measurement of glucose and palmitate oxidation under hypothermic conditions accurately reflects the overall change in energy metabolism occurring as a result of hypothermia.

Changes in High Energy Phosphate Levels

AMP, ADP and ATP levels in hearts frozen at the end of the control, hypothermia and rewarmed periods are shown in Table IV.9, while levels of creatine and creatine phosphate are shown Table IV.10. AMP and ADP levels tend to decrease in both species during hypothermia, although only the value for the ADP level in rat hearts decreases significantly from normothermic control period values. Following rewarming both AMP and ADP levels in rat hearts are significantly higher than levels observed in ground squirrel hearts. At the end of the control period, the ATP level in rat hearts is significantly lower than that observed in ground squirrel hearts; however, no species differences are noted for the hypothermic or rewarmed periods. At the end of the rewarmed period, the ATP level in both species is significantly depressed from control period values.

Creatine and creatine phosphate levels (Table IV.10) do not vary significantly between species during the control or hypothermia period, with the exception of creatine levels which are significantly higher in ground squirrels during the control period. Creatine levels at the end of the rewarmed period are significantly lower than pre-hypothermia values in both species. Levels of creatine phosphate are only significantly lower in rat hearts at the end of the rewarmed period. As ATP and creatine phosphate levels are not significantly depressed during hypothermia, this data suggests that hearts from either species are not metabolically compromised during hypothermia. The poor recovery of function in rat hearts following rewarming, may have affected the levels of high energy phosphates by the end of the rewarmed period (200 min), as AMP and

ADP levels are higher, and the creatine phosphate level is lower than that observed in ground squirrel hearts.

Changes in Glycogen and Triacylglycerol Levels.

Glycogen and triacylglycerol levels from hearts frozen at the end of the control, hypothermia and rewarmed periods are shown in Table IV.11. Although ground squirrels had lower glycogen and higher triacylglycerol levels than the rats, the levels of these substrates did not vary significantly in either species over the course of the experiment. As a result, endogenous energy stores were not preferentially mobilized in either species as a result of hypothermia and rewarming. This suggests that the variations in exogenous energy substrate metabolism observed over the course of the experimental protocol cannot be explained by the preferential utilization of endogenous substrates.

Table IV. 1

Comparison of heart rate, peak systolic pressure and cardiac power between ground squirrels and rats.

	Heart Rate (beats·min^{-1})	PSP (mm Hg)	Cardiac Power (mm Hg·ml·min^{-1}·10^{-2})
Control			
Ground Squirrel	260 ± 15	111.0 ± 5.4	55.9 ± 7.7
Rat	225 ± 7	120.4 ± 3.2	52.4 ± 3.8
Hypothermia			
Ground Squirrel	40 ± 4*	83.2 ± 7.1*	19.6 ± 4.4*
Rat	32 ± 1*	93.6 ± 1.9*	8.3 ± 0.6†*
Rewarmed			
Ground Squirrel	295 ± 15	101.2 ± 4.7	51.2 ± 6.7
Rat	244 ± 8	98.4 ± 3.2*	19.1 ± 3.6†*

Values are the mean ± S.E.M. of 16 ground squirrel hearts and 17 rat hearts. PSP, peak systolic pressure.
†, denotes significantly different between rats and ground squirrels.
*, denotes significantly different from respective control period values.

Table IV.2

Comparison of coronary flow, cardiac output and coronary flow normalized for cardiac power between ground squirrels and rats.

	Coronary Flow $(ml \cdot min^{-1})$	Cardiac Output $(ml \cdot min^{-1})$	CF/ CP $(mm\ Hg^{-1} \cdot 10^{2})$
Control			
Ground Squirrel	32.8 ± 2.8	48.1 ± 5.6	0.76 ± 0.08
Rat	17.8 ± 1.0†	43.4 ± 2.9	0.54 ± 0.14
Hypothermia			
Ground Squirrel	17.9 ± 2.9*	21.1 ± 3.9*	1.38 ± 0.16*
Rat	8.8 ± 0.7†*	8.8 ± 0.7†*	1.06 ± 0.03*
Rewarmed			
Ground Squirrel	33.6 ± 3.5	48.3 ± 5.4	0.83 ± 0.11
Rat	14.1 ± 1.2†*	21.1 ± 2.6†*	0.81 ± 0.08

Values are the mean ± S.E.M. of 16 ground squirrel hearts and 17 rat hearts. CF/CP, coronary flow normalized for cardiac power.

†, denotes values which are significantly different between rats and ground squirrels.

*, denotes values which are significantly different from respective control values.

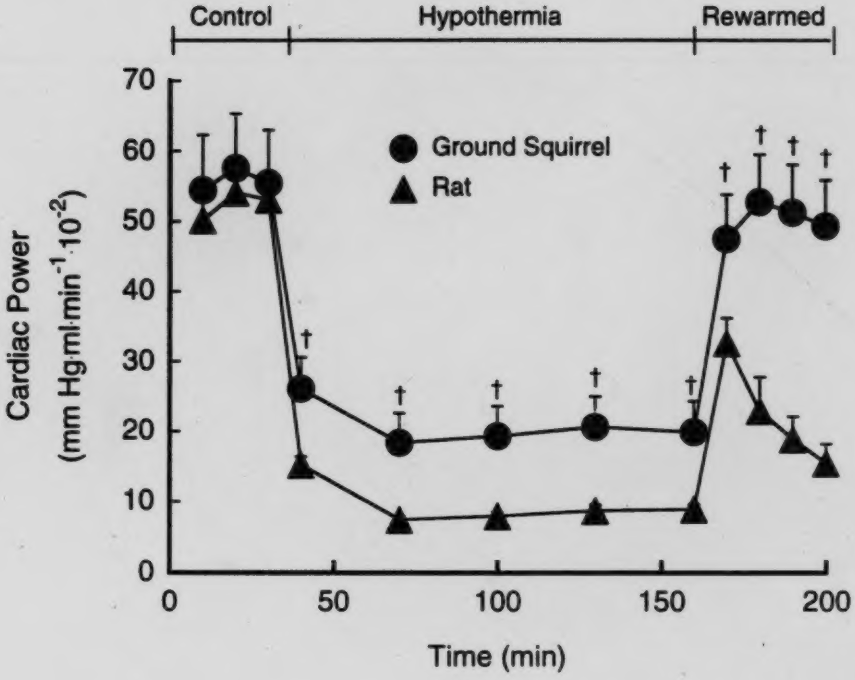

Figure IV.1 Cardiac power in isolated working hearts from ground squirrels and rats over the course of the hypothermia-rewarming perfusion protocol. Values represent mean ± S.E.M. of 16 ground squirrel hearts and 17 rat hearts.
†, significantly different between species at the same time point.

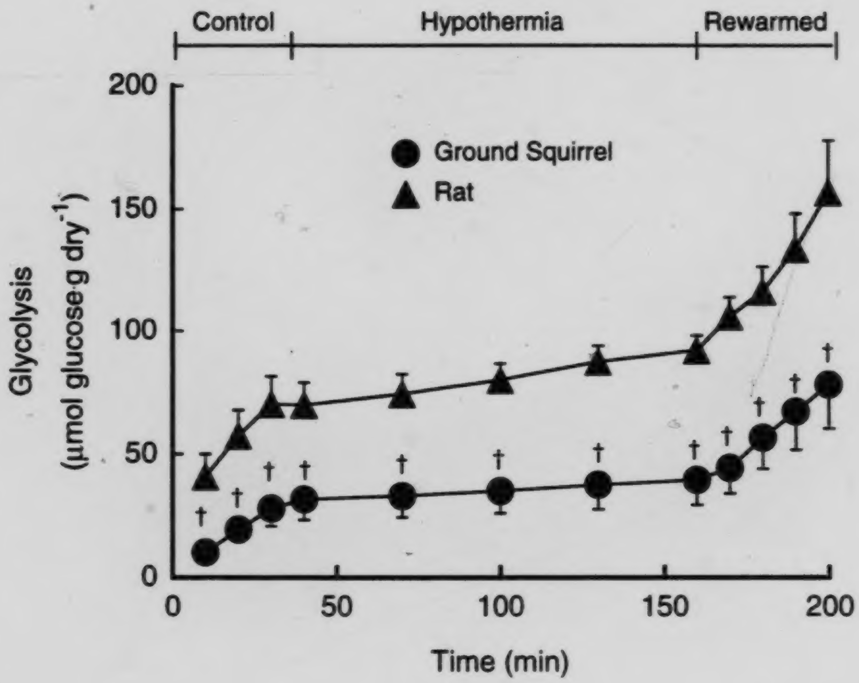

Figure IV.2 Cumulative changes in glycolysis over the course of the experimental protocol. Values represent mean ± S.E.M. For 7 ground squirrel hearts and 8 rat hearts.

†, significantly different between species at the same time point.

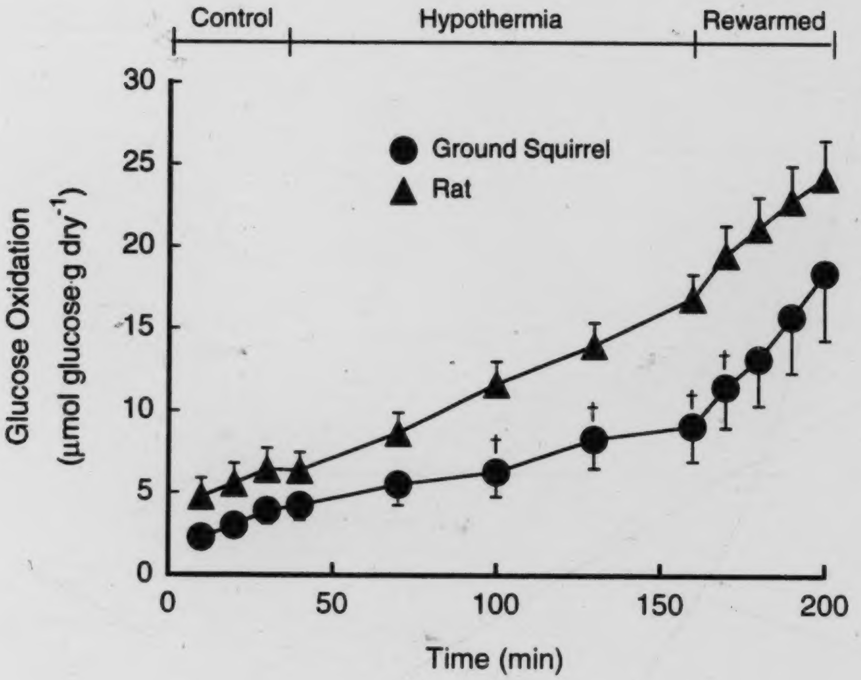

Figure IV.3 Cumulative changes in glucose oxidation over the course of the experimental protocol. Values represent mean ± S.E.M. for 7 ground squirrel hearts and 8 rat hearts.

†, significantly different between species at the same time point.

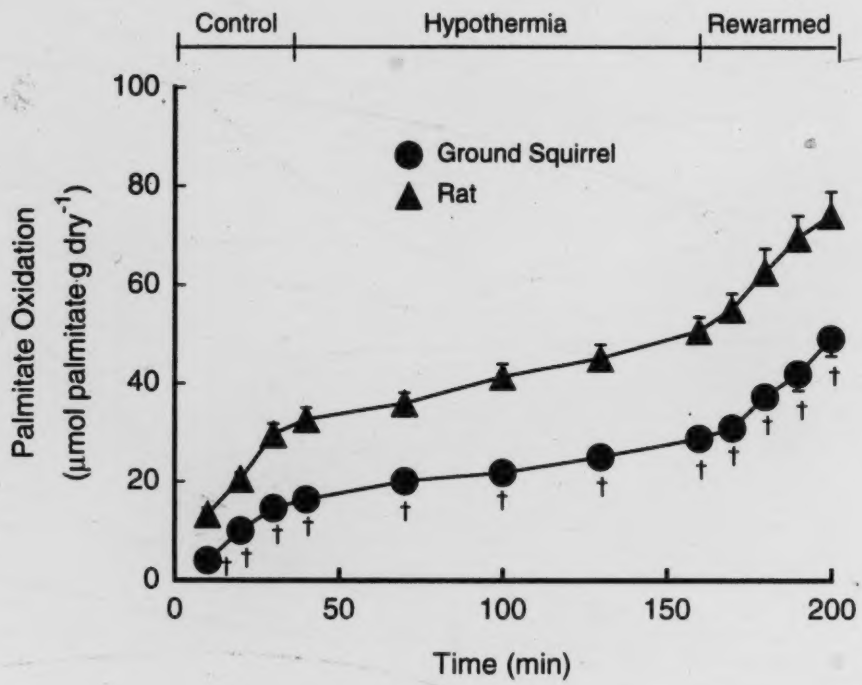

Figure IV.4 Cumulative changes in palmitate oxidation over the course of the experimental protocol. Values represent mean ± S.E.M for 9 ground squirrel hearts and rat hearts each.

†, significantly different between species at the same time point.

Table IV.3

The effects of hypothermia and rewarming on the steady state rates of glycolysis, glucose oxidation and palmitate oxidation in hearts from ground squirrels and rats.

	Glycolysis	Glucose Oxidation	Palmitate Oxidation
		(nmol · g dry^{-1}·min^{-1})	
Control			
Ground Squirrel	887 ± 270	81 ± 20	529 ± 78
Rat	1459 ± 185	89 ± 15	796 ± 50†
Hypothermia			
Ground Squirrel	58 ± 16*	46 ± 11	102 ±14*
Rat	189 ± 53*	88 ± 8†	150 ± 15†*
Rewarmed			
Ground Squirrel	1127 ± 258	232 ± 57*	612 ± 64
Rat	1773 ± 695	168 ± 24*	674 ± 50

Values are the mean ± S.E.M. of 7 ground squirrel and 8 rats hearts used to measure glycolysis and glucose oxidation, and 9 ground squirrel and rat hearts used to measure palmitate oxidation.
†, denotes values which are significantly different between rats and squirrels.
*, denotes values which are significantly different from respective control values.

Table IV.4

The effects of hypothermia and rewarming on rates of substrate metabolism normalized for differences in cardiac power in ground squirrel and rat hearts.

	Glycolysis	Glucose Oxidation	Palmitate Oxidation
		(nmol·mm Hg^{-1}·ml^{-1}·10^{2})	
Control			
Ground Squirrel	19.5 ± 12.8	1.0 ± 0.3	4.2 ± 0.3
Rat	7.2 ± 1.3	0.4 ± 0.1	4.9 ± 0.6
Hypothermia			
Ground Squirrel	6.7 ± 2.5	3.4 ± 1.1*	2.4 ± 0.5*
Rat	5.3 ± 1.6	2.8 ± 0.4*	5.9 ± 0.8†
Rewarmed			
Ground Squirrel	29.7 ± 15.1	3.8 ± 0.9*	4.7 ± 0.5
Rat	19.5 ± 6.1	2.2 ± 0.7*	17.7 ± 2.7†*

Values represent mean ± S.E.M. of 7 ground squirrel and 8 rats hearts used to measure glycolysis and glucose oxidation, and 9 ground squirrel and rat hearts used to measure palmitate oxidation.
†, denotes values significantly different between rats and squirrels.
*, denotes values significantly different from respective control period values.

Table IV.5

Glucose oxidation and glycolysis during rewarming.

	Glucose Oxidation	**Glycolysis**
	($nmol \cdot min^{-1} \cdot g\ dry^{-1}$)	
Ground Squirrel	204 ± 58	467 ± 94
Rat	254 ± 59	1567 ± 293†

Values represent mean ± S.E.M for n = 7 ground squirrels and n = 8 rats.
†, denotes values significantly different between rats and squirrels.

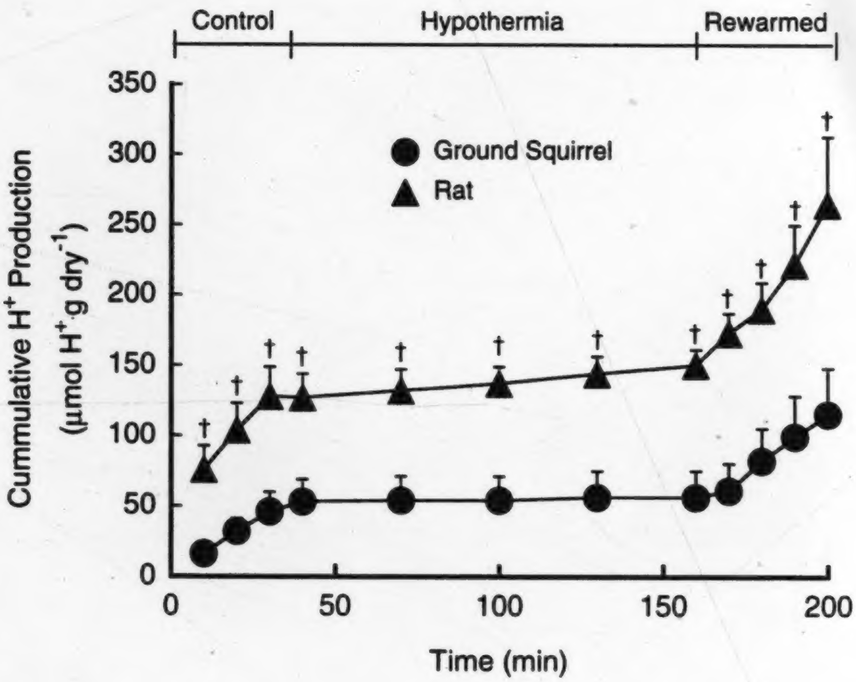

Figure IV.5 Cumulative production of H+ from glucose metabolism over the course of the experimental protocol in ground squirrel and rat hearts. Values represent mean ± S.E.M. for 7 ground squirrel hearts and 8 rat hearts †, significantly different between species at the same time point.

Table IV.6

H^+ production from the uncoupling of glycolysis and glucose oxidation in ground squirrel and rat hearts.

	Control	Hypothermia	Rewarmed	
		($nmol\ H^+ \cdot min^{-1} \cdot g\ dry^{-1}$)	(160-170 min)	(170-200 min)
Ground Squirrel	1613 ± 524	49 ± 28	646 ± 191	1791 ± 479
Rat	2742 ± 344	248 ± 103	2625 ± 561†	3155 ± 1361

Values represent mean ± S.E.M for 7 ground squirrels and 8 rats.

†, denotes values which are significantly different between rats and squirrels.

Table IV.7

Contribution of glucose and palmitate to acetyl-CoA production in ground squirrel and rat hearts.

	Glucose Oxidation		Palmitate Oxidation		Total Production
	(nmol Acetyl-CoA·g dry^{-1}·min^{-1})				
Control					
Ground Squirrel	0.16 ± 0.04	(4%)	4.23 ± 0.62	(96%)	4.39 ± 0.62
Rat	0.18 ± 0.03	(3%)	6.37 ± 0.40†	(97%)	6.55 ± 0.40†
Hypothermia					
Ground Squirrel	0.09 ± 0.02	(11%)	0.82 ± 0.11*	(89%)	0.91 ± 0.11*
Rat	0.18 ± 0.02†	(13%)	1.20 ± 0.12†*	(87%)	1.37 ± 0.12†*
Rewarmed					
Ground Squirrel	0.46 ± 0.11*	(9%)	4.90 ± 0.51	(91%)	5.36 ± 0.51
Rat	0.34 ± 0.05*	(6%)	5.39 ± 0.40	(94%)	5.72 ± 0.40

Acetyl-CoA production rates were calculated from the rates of glucose oxidation and palmitate oxidation shown in Table IV.3. Values in parenthesis represent the percent contribution to total acetyl-CoA production from exogenously metabolized substrates.
†, significantly different between rats and squirrels.
*, significantly different from respective control values.

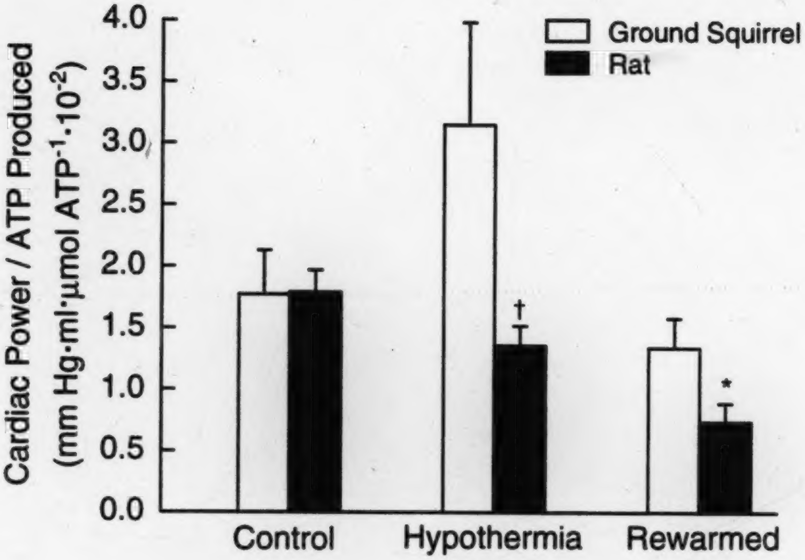

Figure IV.6 The efficiency of converting ATP production from substrate metabolism into cardiac power in ground squirrel and rat hearts.
*, significantly different from initial control values.
†, significantly different between species during the same phase of the protocol.

Table IV.8

O_2 consumption and cardiac efficiency measured at 37° C and 15° C in ground squirrel and rat hearts.

	Cardiac Power (mm Hg·ml·min^{-1}·10^{-2})	O_2 Consumption (µmol O_2·min^{-1})	Cardiac Efficiency (mm Hg·ml·µmol O_2^{-1}·10^{-2})
Ground Squirrel			
37° C	68.2 ± 17.6	16.6 ± 1.4	4.0 ± 0.7
15° C	18.1 ± 7.5*	3.0 ± 1.3*	5.6 ± 1.0*
Rat			
37° C	67.7 ± 7.3	14.5 ± 0.6	4.7 ± 0.5
15° C	11.1 ± 2.1*	3.5 ± 0.5*	3.2 ± 0.3*

Values represent mean ± S.E.M. for 10 rat hearts and 4 ground squirrel hearts. *, denotes values which are significantly different between 37° C and 15° C.

Table IV.9

Adenine nucleotide levels in ground squirrel and rat hearts.

	AMP	ADP	ATP
		($\mu mol \cdot g\ dry^{-1}$)	
Control			
Ground Squirrel	2.3 ± 0.9	14.3 ± 6.2	34.2 ± 3.2
Rat	2.8 ± 0.4	15.1 ± 0.4	25.6 ± 1.6†
Hypothermia			
Ground Squirrel	1.4 ± 0.4	10.4 ± 1.5	26.9 ± 5.4
Rat	2.3 ± 0.3	9.3 ± 1.0*	23.5 ± 2.5
Rewarmed			
Ground Squirrel	1.3 ± 0.2	10.2 ± 0.9	21.7 ± 1.6*
Rat	3.0 ± 0.2†	13.1 ± 0.7†*	18.6 ± 0.9*

Values are the mean ± S.E.M. of 6, 8 and 9 ground squirrel hearts frozen at the end of the control hypothermia and rewarmed periods, respectively, and 6 rats hearts frozen at the end of each time period.
†,significantly different between rats and ground squirrels.
*, significantly different from respective control period values.

Table IV.10

Creatine and creatine phosphate levels in ground squirrel and rat hearts.

	Creatine	Creatine Phosphate
	($\mu mol \cdot g\ dry^{-1}$)	
Control		
Ground Squirrel	123.8 ± 7.5	60.8 ± 4.9
Rat	95.2 ± 4.1†	50.7 ± 3.9
Hypothermia		
Ground Squirrel	86.5 ± 5.8*	61.5 ± 6.7
Rat	77.2 ± 8.4	54.2 ± 9.8
Rewarmed		
Ground Squirrel	82.4 ± 3.2*	52.2 ± 4.8
Rat	77.6 ± 3.3*	30.3 ± 2.8†*

Values are the mean ± S.E.M. of 6, 8 and 9 ground squirrel hearts frozen at the end of the control hypothermia and rewarmed periods, respectively, and 6 rats hearts frozen at the end of each time period.
†,significantly different between rats and ground squirrels.
*, significantly different from respective control period values.

Table IV.11

Glycogen and triacylglycerol levels of ground squirrel and rat hearts frozen at the end of the control, hypothermia and rewarmed periods.

	Glycogen *(μmol glucose·g dry^{-1})*	Triacylglycerol *(μmol·g dry^{-1})*
Control		
Ground Squirrel	98.8 ± 7.7	39.0 ± 4.5
Rat	122.8 ± 12.6	20.5 ± 2.0†
Hypothermia		
Ground Squirrel	91.8 ± 3.4	35.5 ± 5.6
Rat	116.9 ± 10.2†	20.9 ± 2.9†
Rewarmed		
Ground Squirrel	88.4 ± 7.9	43.2 ± 7.5
Rat	128.0 ± 8.6†	24.6 ± 2.5†

Values are the mean ± S.E.M. of 6 ground squirrel and rat hearts frozen at the end of the control period and hypothermia periods, 8 ground squirrel and rat hearts frozen at the end of the rewarmed period.
†, denotes values which are significantly different between rats and squirrels.

Discussion

This study compared energy substrate metabolism and cardiac function in a cold-sensitive species (rat) and a cold-tolerant species (Richardson's ground squirrel) under conditions of hypothermia and rewarming. The superior performance of ground squirrel hearts relative to rat hearts during hypothermia confirms previous observations made between these species (Burlington and Darvish, 1988; Burlington et al, 1989; Caprette and Senturia, 1984). Our results expand on these previous observations by showing that the recovery of function following rewarming was significantly better in ground squirrel hearts than rat hearts despite the presence of 1.2 mM palmitate. This suggests that the altered physiological state occurring in rat hearts as a result of hypothermia-rewarming, which makes them susceptible to the depressive effects of 1.2 mM palmitate, does not occur in ground squirrel hearts.

Despite this difference in cardiac performance, hypothermia had a similar general effect on substrate metabolism in both species, although it did not affect all metabolic pathways equally. Hypothermia is capable of altering energy substrate metabolism both by reducing metabolic demand, through a decrease in cardiac power output, and by directly affecting the activity of enzymes within the various metabolic pathways. In both species the general order in which hypothermia depressed substrate metabolism was glycolysis > palmitate oxidation > glucose oxidation. Although ATP derived from glycolysis is thought to be used preferentially for ion homeostasis (Glitch and Tape, 1993; Xu et al., 1995), the similar decrease in glycolysis in both species as a result of hypothermia cannot explain the poor performance of rat hearts during the hypothermia period. Similarly, the low glycolytic rate coupled with the maintenance of ATP and creatine phosphate levels observed in rat and ground squirrel hearts during hypothermia indicates that the decrease in coronary flow was not limiting function or energy substrate metabolism during hypothermia (i.e. hearts were not ischemic). Thus, although previous studies have suggested that rat hearts lose the ability to regulate coronary flow at low temperatures (Burlington et al., 1989), this does not appear to limit oxidative metabolism. Following rewarming, substrate metabolism returns to pre-hypothermic values in both species, indicating that the poor recovery of function in rat hearts cannot be explained as the result of an inability to metabolize energy yielding substrates.

When substrate metabolism is examined as a function of the level of cardiac power output being performed by hearts, major species differences in oxidative metabolism appear during hypothermia. In both species, the contribution of glucose oxidation to cardiac work is increased. In contrast, the contribution of palmitate oxidation decreases significantly in ground squirrel hearts but not rat hearts. As the oxidation of glucose and fatty acids are reciprocally regulated (Randle et al., 1994; Sugden and Holness, 1994), this observation implies that this regulation is intact in the hearts of ground squirrel at low temperatures, but may not occur in rat hearts, based on the observation that palmitate oxidation per unit work does not decrease in rat hearts. The differences in energy substrate metabolism between species, or those which arise within a species as a result of hypothermia and rewarming, cannot be explained by the preferential utilization of endogenous substrates (glycogen and triacylglycerol), as the level of these substrates did not vary significantly over the course of the experiment.

The major species differences observed in this study were related to H^+ production as a result of the uncoupling between glycolysis and glucose oxidation, and the efficiency of converting energy substrates into cardiac power during hypothermia and following rewarming. H^+ production was depressed in both species during hypothermia as the result of a tighter coupling between glycolysis and glucose oxidation. The rate of H^+ production did not differ significantly between the species over the course of the experiment with the exception of rewarming (160-170 min), where the level of H^+ production was significantly higher in rat than ground squirrel hearts. This effect was due primarily to species differences in the recovery of glycolysis during the period of rewarming.

The other major species difference dealt with the efficiency of converting energy substrate metabolism into cardiac power during hypothermia and rewarming. During hypothermia the level of cardiac work obtained from the level of metabolism increased in ground squirrel hearts but decreased in rat hearts so that the species differences in cardiac efficiency were significant. This effect was observed in another series of hearts in which O_2 consumption was used as an index of metabolism. The main reason for this difference in cardiac efficiency appears to be due to a decrease in the oxidation of palmitate (normalized for cardiac work), in ground squirrel hearts (but not rat hearts), under hypothermic conditions. Whether the decrease in palmitate oxidation contributes directly to the improved functional recovery observed in ground squirrels, or is simply a production of the improved efficiency of the ground squirrel heart remains to be established. Following rewarming cardiac efficiency returns to pre-hypothermic values in ground squirrel hearts but is significantly depressed in rat hearts.

In summary, this study was able to confirm previous observations that ground squirrel hearts function better under hypothermic conditions than rat hearts. Similarly, the recovery of heart function following rewarming is significantly better in ground squirrel hearts than rat hearts. When these hearts were examined for energy substrate metabolism, the same general pattern of substrate metabolism over the course of the hypothermia-rewarming protocol was observed in both species, namely that glycolysis was depressed to the greatest extent, while glucose oxidation was least affected by hypothermia. However, when energy substrate metabolism was normalized for differences in cardiac power output, species differences in oxidative metabolism were observed. In both species, hypothermia was accompanied by an increase in the contribution of glucose oxidation toward cardiac power output. While this was accompanied by a decrease in the contribution of palmitate oxidation in ground squirrel hearts, it did not occur in rat hearts. As a result, the efficiency of converting energy substrates into cardiac power was higher in ground squirrel hearts than rat hearts during hypothermia. This higher level of efficiency was also observed in ground squirrel hearts following rewarming. H^+ production was similar between the two species with the exception of rewarming, where a higher rate of glycolysis led to a higher rate of H^+ production which could contribute towards the species differences in functional recovery following rewarming. Endogenous substrate utilization does not play a role in mediating the effects of hypothermia-rewarming for the two groups.

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Chapter V

The Effects of Calcium on Function and Metabolism During Hypothermia and Rewarming in the Rat and Richardson's Ground Squirrel Heart

Introduction

Results from the two previous studies have indicated that: 1) fatty acids are capable of depressing cardiac function following hypothermia-rewarming, 2) this effect is limited to cold-sensitive species, 3) cardiac efficiency is lower in cold-sensitive species than cold-tolerant species during hypothermia, and 4) the poor recovery of function during rewarming is not associated with a reduction in energy substrate metabolism. Hypothermia is known to be associated with a greater rise in cytosolic Ca^{2+} in rat myocytes than ground squirrel myocytes (Liu et al., 1991, Liu and Wang, 1993). Under conditions of hypothermic storage, a rise in intracellular Ca^{2+} is thought to be detrimental, leading to the depletion of energy stores, and the disruption of normal myocyte physiology (Robinson and Harwood, 1991; Stringham et al., 1992; Rebeyka et al., 1990). This implies that a rise in intracellular Ca^{2+} in rat hearts during hypothermia may be responsible for the lower cardiac efficiency observed in this species during hypothermia, and the poor recovery of function following rewarming.

The decrease in cardiac efficiency observed in rat hearts is analogous to the phenomenon of stunning observed following ischemia-reperfusion, where the depression in mechanical function is not associated with a parallel reduction in O_2 consumption (Kusuoka et al., 1990). Myocardial stunning as a result of ischemia-reperfusion is associated with a decrease in the activity of the sarcoplasmic reticulum (SR), and the development of a Ca^{2+} overload (Mentzer, 1993; Mitchell et al., 1993). As with ischemia, hypothermia may also lead to a reduction in sarcoplasmic reticulum function, which may contribute to the stunning like phenomenon observed following rewarming (Labow et al., 1993; Liu et al., 1993, 1994). The fact that ground squirrel hearts are unaffected by low temperature may be related to the recent observation that SR activity is inherently higher in ground squirrel hearts than rat hearts, and is more resistant to inhibition by low temperature than rat hearts (Liu et al., 1996). Although a rise in intracellular Ca^{2+} is detrimental under hypothermic conditions involving ischemia, where ATP production is limited (Stringham et al., 1991), the role that any increase in cytosolic Ca^{2+} plays in mediating the poor recovery in function following rewarming in the absence of ischemia is unknown. Steigen et al (1994) have suggested that the poor recovery of function following rewarming in the presence of 1.2 mM palmitate is associated with an increased level of myocardial tissue Ca^{2+}.

Although Ca^{2+} may be detrimental by stimulating an increased wastage of energy, leading to a decrease in cardiac efficiency, Ca^{2+} may play a beneficial role in hearts perfused with high concentrations of fatty acids by stimulating glucose oxidation. Increasing extracellular Ca^{2+} has been shown to stimulate glucose oxidation (Schonekess et al., 1995), secondary to the stimulation of PDC (McCormack and Denton, 1989). As the stimulation of glucose oxidation has been shown to be beneficial in improving the recovery of function following ischemia-reperfusion in the presence of high concentrations of fatty acids (Lopaschuk et al., 1993; Lewandowski and White, 1995, Broderick et al., 1993), Ca^{2+} can potentially induce effects which may be either deleterious or beneficial to the recovery of function by increasing energy expenditure during hypothermia, and increasing glucose oxidation during rewarming.

In this study, rat and ground squirrel hearts were subjected to a hypothermia-rewarming protocol in the presence of 1.2 mM palmitate. In order to examine the effects of Ca^{2+} on cardiac performance, hearts were perfused with either 1.25 or 2.50 mM Ca^{2+}. Ground squirrel hearts were used to provide a comparison between the effects of altering perfusate Ca^{2+} on a cold-sensitive species and a cold-tolerant one. This study examines the effect of altering perfusate Ca^{2+} concentration on cardiac efficiency during hypothermia and rewarming to determine if Ca^{2+} plays a role in the decrease in cardiac efficiency observed in rat hearts, and to determine if this plays a role in mediating the poor recovery of function observed in rat hearts following rewarming from hypothermia.

Methods

Animals

The animals used in this study were Sprague-Dawley rats and Richardson's ground squirrels (*Spermophilus richardsonii*). Ground squirrels used in this study were all in the non-hibernating phase of their annual hibernation cycle.

Heart Perfusions

Isolated working heart perfusions were performed as outlined in Chapter II. The hypothermia-rewarming perfusion protocol was also as described in Chapter II.

Perfusion Conditions

The Krebs'-Henseleit solution used in the experiment contained either 1.25 or 2.50 mM free Ca^{2+} along with 11 mM glucose and 1.2 mM palmitate. Hearts from both species were perfused according to the hypothermia protocol outlined in Chapter II. Hearts were frozen at the end of the control, hypothermic and rewarmed periods for analysis of endogenous substrate (glycogen and triacylglycerol) levels.

Measurement of Glycolysis, Glucose Oxidation and Palmitate Oxidation

As outlined in Chapter II, glycolysis and glucose oxidation were measured simultaneously in the working hearts by perfusing with [5-^{3}H/U-^{14}C]glucose. Palmitate oxidation was measured in a separate series of hearts perfused with [9,10-^{3}H] palmitate. The contribution of glucose and palmitate to supplying acetyl-CoA to the TCA cycle was calculated by using the stoichiometric ratio of 2 mol acetyl-CoA/mol glucose and 8 mol acetyl-CoA/mol palmitate. Hydrogen ion

(H^+) production from the imbalance between glycolysis and glucose oxidation was calculated for each heart by subtracting the rate of glucose oxidation from glycolysis and multiplying the resulting number by two as outlined by Lopaschuk et al (1993).

Determination of Glycogen and Triacylglycerol levels

Glycogen and triacylglycerol levels were determined as outlined in Chapter II.

Statistical Analysis

A Single Factor Analysis of Variance with a Student-Neuman-Keuls test was used to determine if values were significantly different between control, hypothermia and rewarmed periods within a group, while an unpaired t-test was used when making comparisons between groups perfused with different Ca^{2+} concentrations. A value of $p < 0.05$ was considered to be significant. All values presented represent the mean ± S.E.M.

RESULTS

Mechanical Function

Rats

The effects of hypothermia and rewarming on heart rate and peak systolic pressure are shown in Table V.1, while values for coronary flow, cardiac output and cardiac power are shown in Table V.2. During the initial normothermic control period perfusion, none of the functional parameters were significantly different between rat hearts perfused with 1.25 or 2.50 mM Ca^{2+}. Hypothermia led to a significant reduction in all functional parameters regardless of perfusate Ca^{2+} concentration. Perfusing with 2.50 mM Ca^{2+} led to a significant decrease in peak systolic pressure and cardiac power during the hypothermic perfusion. Following rewarming, peak systolic pressure was significantly lower than control period values in both groups of rat hearts. The recovery of function following rewarming differed significantly between the groups. In the 1.25 mM Ca^{2+} group, coronary flow, cardiac output and cardiac power were all significantly lower than control period values. These values were also significantly lower than comparable values obtained from the 2.50 mM Ca^{2+} group during the rewarmed period.

Changes in cardiac power over the course of the course of the hypothermia-rewarming protocol is shown in Figure V.1. In both groups, the level of cardiac power obtained from the hearts was constant over the 2 hr hypothermic perfusion. During the hypothermia period, the level of cardiac power obtained from the 1.25 mM Ca^{2+} group was significantly higher than in the 2.50 mM Ca^{2+} group. In contrast, the level of cardiac power in the 2.50 mM Ca^{2+} group was significantly higher than the 1.25 mM Ca^{2+} group at each time point during the rewarmed period. For both groups, the highest level of cardiac power was obtained immediately following rewarming (170 min), with cardiac power declining in both groups over the rewarmed period.

Ground Squirrels

The average values for heart rate and peak systolic pressure are shown in Table V.1, while values for coronary flow, cardiac output and cardiac power are shown in Table V.2. While hypothermia led to a significant depression in all parameters of mechanical function in ground squirrel hearts, all parameters returned to control period levels during the rewarmed period. Increasing perfusate Ca^{2+} concentration from 1.25 to 2.50 mM Ca^{2+} did not significantly affect any of the parameters during either the normothermic (control, rewarmed) or hypothermic perfusion periods. The changes in cardiac power over the course of the experiment are shown in Figure V.2. The only significant difference in cardiac power between the two groups occurred following the initial cooling of the hearts at the 40 minute time point, where the 2.50 mM Ca^{2+} group was significantly lower than the 1.25 mM Ca^{2+} group. Apart from this difference cardiac power output was not significantly different between the groups over the course of the experiment, and the level of cardiac power obtained from the hearts was constant during the hypothermia and rewarmed periods.

Energy Substrate Metabolism

Glycolysis

The steady state rate of glycolysis measured during the normothermic and hypothermic periods for both species is shown in Figure V.3. The rate of glycolysis was significantly reduced during hypothermia in both species regardless of perfusate Ca^{2+} concentration. Glycolytic rates were severely depressed in both species during hypothermia being only 7-12% of the control period values. The rate of glycolysis tended to be higher in rat hearts perfused with 2.50 mM than 1.25 mM Ca^{2+} during the normothermic (control, rewarmed) perfusion periods, although this difference was not significant. Glycolytic flux returned to control period values upon rewarming for both groups of rat hearts.

Increasing perfusate Ca^{2+} concentration had no effect on glycolytic rate in ground squirrel hearts during either the normothermic or hypothermic perfusion periods. Glycolytic rate in ground squirrel hearts was generally lower than that observed in rat hearts throughout the hypothermia-rewarming protocol.

Glucose Oxidation

The steady state rate of glucose oxidation for the various groups is shown in Figure V.4. In contrast to glycolysis, glucose oxidation was not significantly depressed from control period values in either species as a result of hypothermia. In rat hearts perfused with 2.5 mM Ca^{2+}, the rate of glucose oxidation during the normothermic perfusion periods was significantly higher than hearts perfused with 1.25 mM Ca^{2+}. Glucose oxidation during hypothermia also tended to be higher in the 2.50 mM Ca^{2+} group, although this difference was not significant. Following rewarming, the rate of glucose oxidation in both groups of rat hearts recovered to a higher value than that observed during the control period.

While perfusing ground squirrel hearts with 2.50 mM Ca^{2+} led to a small increase in the rate of glucose oxidation over the entire perfusion protocol, it was not significantly greater than the rate obtained with 1.25 mM Ca^{2+}.

Palmitate Oxidation

The steady state rate of fatty acid oxidation for the various groups is shown in Figure V.5. For both species, hypothermia led to a significant reduction in the rate of palmitate oxidation relative to control period values. In rat hearts, increasing perfusate Ca^{2+} concentration from 1.25 to 2.50 mM led to a significant reduction in the rate of palmitate oxidation over all periods of the experimental protocol. Following rewarming, the rate of palmitate oxidation was depressed relative to control period values in rat hearts perfused with 1.25 mM Ca^{2+}. Palmitate oxidation returned to pre-hypothermic levels in hearts perfused with 2.50 mM Ca^{2+}. This may be due, in part, to the poorer recovery of function during the rewarmed period in rat hearts perfused with 1.25 mM Ca^{2+}, leading to a decrease in metabolic demand.

In contrast, the rate of palmitate oxidation was only significantly depressed in ground squirrel hearts perfused with 2.50 mM Ca^{2+} during the hypothermic perfusion, with no significant difference being observed between groups during the control or rewarmed perfusion periods.

Glycolysis and Glucose Oxidation During Rewarming

Glycolysis and glucose oxidation during rewarming (160-170 min) are shown in Figure V.6. These values cannot be considered as a true steady state rate due to the influence of increasing temperature and cardiac power output on substrate metabolism during rewarming. Although glycolytic rate was similar in rat hearts perfused with different Ca^{2+} concentrations, the rate of glucose oxidation was significantly higher in rat hearts perfused with 2.50 mM Ca^{2+}. In contrast, glycolysis and glucose oxidation was similar in ground squirrel hearts perfused with either Ca^{2+} concentration.

H^+ Production

Hydrogen ion (H^+) production resulting from the imbalance between glycolysis and glucose oxidation is shown in Table V.3. In all groups, H^+ production is dramatically reduced as a result of hypothermia, with rates decreasing approximately 14 and 36 fold for rat hearts perfused with 1.25 and 2.50 mM Ca^{2+}, respectively, and 47 and 53 fold in ground squirrel hearts under the same conditions. H^+ production returned to control period values in both species upon rewarming regardless of Ca^{2+} concentration. Despite changes in H^+ production for the different periods of the perfusion protocol, changing perfusate Ca^{2+} concentration did not significantly affect the rates of H^+ production for either species. However, during rewarming (160-170 min) the rate of H^+ production was significantly lower than control period values in rat hearts perfused with 2.50 mM Ca^{2+}, but not in hearts perfused with 1.25 mM Ca^{2+}. This effect was primarily the result of a greater recovery of glucose oxidation during rewarming in rat hearts perfused with 2.50 mM Ca^{2+}.

TCA Acetyl-CoA Production From Energy Substrates

The total acetyl-CoA for the TCA cycle derived from glucose and palmitate metabolism is shown in Figure V.7. In both species total acetyl-CoA derived from substrate metabolism is significantly decreased during hypothermia, but returns to pre-hypothermia levels following rewarming. In rat hearts perfused with 2.50 mM Ca^{2+}, the level of acetyl-CoA production during the control and hypothermia periods is significantly lower than in hearts perfused with 1.25 mM Ca^{2+}. In contrast, changing perfusate Ca^{2+} concentration did not alter total acetyl-CoA production in ground squirrel hearts under either normothermic or hypothermic conditions.

The individual contribution of glucose and palmitate to acetyl-CoA production normalized for cardiac power for the control and hypothermia periods is shown in Figure V.8. For both species, the contribution of glucose to acetyl-CoA formation (Figure V.8A) increases during hypothermia regardless of perfusate Ca^{2+} concentration. Increasing perfusate Ca^{2+} led to a significant increase in the contribution of glucose to acetyl-CoA formation in rat hearts during both the control and hypothermic period. In contrast, increasing perfusate Ca^{2+} had no effect on ground squirrel hearts.

In contrast to glucose oxidation, the effects of hypothermia and Ca^{2+} concentration on palmitate metabolism varied greatly among the groups (Figure V.8B). The contribution of palmitate to acetyl-CoA production during the control period was significantly lower in rat hearts perfused with 2.50 mM Ca^{2+} than in rat hearts perfused with 1.25 mM Ca^{2+}. During the hypothermic perfusion period, the contribution from palmitate increased significantly in rat hearts perfused with 2.50 mM Ca^{2+}. In contrast, the contribution of palmitate from rat hearts perfused with 1.25 mM Ca^{2+} increased only slightly as a result of hypothermia.

The results obtained for rat hearts were in contrast to those obtained from ground squirrel hearts, where hypothermia led to a significant reduction in the contribution of acetyl-CoA for palmitate normalized for cardiac power regardless of Ca^{2+} concentration. Changing perfusate Ca^{2+} did not have an effect on the contribution of palmitate derived acetyl-CoA normalized for cardiac power.

Under these conditions, any increase in the contribution of substrate metabolism normalized for cardiac power suggests an increased energy requirement for energy utilizing reactions not involved in cardiac power output, while a decrease represents a reduction energy expenditure. In ground squirrel hearts the increased utilization of glucose is offset by the decreased utilization of palmitate for cardiac work. In rat hearts, the increased utilization of glucose is not offset by a decrease in palmitate. In fact, the significant increase in palmitate in the 2.50 mM Ca^{2+} group as a result of hypothermia, indicates that raising perfusate Ca^{2+} led to a greater requirement for energy which did not contribute to cardiac power output.

The Contribution of Substrate to ATP Production

The relative contribution of glycolysis, glucose oxidation and palmitate oxidation to ATP production in the rat and ground squirrel hearts is shown in Figure V.9. During the initial control period, the majority of ATP in rat hearts was derived from palmitate oxidation (Figure V.9A), although the contribution of glucose metabolism (glycolysis and glucose oxidation) to ATP production was much lower in hearts perfused with 1.25 mM Ca^{2+} than in hearts perfused with 2.50 mM Ca^{2+}. Ground squirrel hearts also predominantly used palmitate oxidation as a source of ATP production, although in these hearts Ca^{2+} concentration had very little effect on the contribution of glucose metabolism to ATP production.

Hypothermia resulted in a dramatic decrease in overall ATP production, as well as in a substantial switch in the relative source of ATP production in all groups. The percent contribution from glucose was increased in all groups due to the fact that glucose oxidation was inhibited the least by lowering heart temperature. This effect was most notable in rat hearts perfused with 2.50 mM Ca^{2+}, where ATP production from glucose utilization accounted for more than a third of total ATP production. During hypothermia palmitate oxidation accounted for 84.4 % of ATP production in rat hearts perfused with 1.25 mM Ca^{2+}, while it was reduced to 63.2% in hearts perfused with 2.50 mM Ca^{2+}.

Following rewarming, the relative contribution of glycolysis, glucose oxidation and palmitate oxidation to ATP production (Figure V.9C) was essentially similar to initial control values. Palmitate oxidation accounted for 88.9% of ATP production in both rat and ground squirrels perfused with 1.25 mM Ca^{2+}, while it accounted for 71.5% and 80.0% of ATP production in rat and ground squirrel hearts perfused with 2.50 mM Ca^{2+}.

Cardiac Efficiency During and Following Hypothermia

The efficiency of translating substrate metabolism into cardiac power during the different periods of the protocol is shown in Table V.4. These values assume the optimal conversion of substrates into ATP. During the control period, the level of efficiency was significantly higher in rat hearts perfused with 2.50 mM than 1.25 mM Ca^{2+}. During hypothermia, the level of efficiency obtained from rat hearts decreased in both groups, but was significantly depressed from control period values in the 2.50 mM Ca^{2+} perfused hearts. During hypothermia cardiac efficiency decreased to 75% of the control period value in 1.25 mM Ca^{2+} rat hearts, and decreased to 40% in 2.50 mM Ca^{2+} rat hearts. Following rewarming, cardiac efficiency continued to decrease in rat hearts perfused with 1.25 mM Ca^{2+}, but increased in hearts perfused with 2.50 mM Ca^{2+}. As a result, efficiency following rewarming was significantly different between these groups. In both groups of rat hearts, the level of efficiency obtained following rewarming was significantly lower than that calculated for the control period.

In contrast, cardiac efficiency increased in ground squirrel hearts during hypothermia, increasing to 178% and 180% of control period values in hearts perfused with 1.25 and 2.50 mM Ca^{2+} respectively. Following rewarming, cardiac efficiency returned to levels which were not significantly different from the control period.

Glycogen and Triacylglycerol Levels.

Endogenous myocardial stores of glycogen and triacylglycerol have the potential to contribute glucose and fatty acids, respectively, as a source of energy during and following hypothermia. We therefore measured glycogen and triacylglycerol levels in hearts frozen at the end of the control, hypothermia and rewarmed periods (Table V.5). Glycogen levels in ground squirrel hearts were generally lower than those observed in rats, although neither hypothermia nor rewarming resulted in a significant depletion of glycogen in rat or ground squirrel hearts perfused with either Ca^{2+} concentration. In fact, glycogen values at the end of rewarming for rat hearts perfused with 2.50 mM Ca^{2+} were significantly increased over initial control values.

Altering perfusate Ca^{2+} concentration did not affect triacylglycerol levels in either species over the course of the experiment. The levels of triacylglycerol were generally higher in ground squirrel hearts than in rat hearts. Triacylglycerol levels measured at the end of hypothermia or rewarming were not significantly different from values observed at the end of the initial control period for any of the groups studied. This suggests that triacylglycerol stores were not preferentially mobilized as a result of either hypothermia or rewarming. This data also suggests that the variations in exogenous palmitate metabolism observed in this study cannot be explained by the preferential utilization of endogenous triacylglycerides.

Table V.1

Heart rate, peak systolic pressure and cardiac power in rat and ground squirrel hearts perfused with different Ca^{2+} concentrations.

	Heart Rate *(beats·min^{-1})*	PSP *(mm Hg)*	Cardiac Power *(mm Hg·ml·min^{-1}·10^{-2})*
Rat			
Control			
1.25 mM Ca^{2+}	224 ± 9	122 ± 4	53.3 ± 4.4
2.50 mM Ca^{2+}	211 ± 8	121 ± 4	53.9 ± 6.3
Hypothermia			
1.25 mM Ca^{2+}	31 ± 2*	93 ± 3*	8.1 ± 0.8*
2.50 mM Ca^{2+}	29 ± 2*	73 ± 3*†	5.2 ± 0.4*†
Rewarmed			
1.25 mM Ca^{2+}	238 ± 8	98 ± 4*	18.9 ± 4.3*
2.50 mM Ca^{2+}	231 ± 7	110 ± 3*	43.4 ± 5.1†
Ground Squirrel			
Control			
1.25 mM Ca^{2+}	257 ± 15	111 ± 7	54.8 ± 8.9
2.50 mM Ca^{2+}	264 ± 16	110 ± 5	55.7 ± 6.7
Hypothermia			
1.25 mM Ca^{2+}	41 ± 5*	85 ± 8*	21.6 ± 5.1*
2.50 mM Ca^{2+}	40 ± 4*	86 ± 5*	17.3 ± 2.8*
Rewarmed			
1.25 mM Ca^{2+}	294 ± 15	101 ± 6	50.5 ± 7.8
2.50 mM Ca^{2+}	300 ± 16	97 ± 5	44.9 ± 6.7

Values are the mean ± S.E.M. for 14 and 21 rat hearts perfused with 1.25 and 2.50 mM Ca^{2+} respectively, and 13 and 17 ground squirrel hearts perfused with 1.25 and 2.50 mM Ca^{2+} respectively.
*, significantly different from respective control period values.
†, significantly different between hearts perfused at different Ca^{2+} concentrations.

Table V.2

Coronary flow and cardiac output from ground squirrel and rat hearts perfused with different Ca^{2+} concentrations.

	Coronary Flow ($ml \cdot min^{-1}$)	Cardiac Output ($ml \cdot min^{-1}$)
Rat		
Control		
1.25 mM Ca^{2+}	18.2 ± 1.0	43.5 ± 3.5
2.50 mM Ca^{2+}	20.2 ± 1.4	41.9 ± 4.1
Hypothermia		
1.25 mM Ca^{2+}	8.3 ± 0.8*	8.3 ± 0.8*
2.50 mM Ca^{2+}	8.3 ± 0.5*	8.3 ± 0.5*
Rewarmed		
1.25 mM Ca^{2+}	13.8 ± 1.2	20.7 ± 3.1*
2.50 mM Ca^{2+}	18.9 ± 1.5†	37.8 ± 3.7†
Ground Squirrel		
Control		
1.25 mM Ca^{2+}	31.9 ± 3.3	46.3 ± 6.2
2.50 mM Ca^{2+}	28.8 ± 3.9	49.9 ± 5.7
Hypothermia		
1.25 mM Ca^{2+}	17.5 ± 3.1*	22.7 ± 4.7*
2.50 mM Ca^{2+}	15.5 ± 2.3*	18.6 ± 2.8*
Rewarmed		
1.25 mM Ca^{2+}	34.3 ± 4.3	47.6 ± 6.3
2.50 mM Ca^{2+}	28.4 ± 3.3	45.9 ± 5.8

Values are the mean ± S.E.M. for 14 and 21 rat hearts perfused with 1.25 and 2.50 mM Ca^{2+} respectively, and 13 and 17 ground squirrel hearts perfused with 1.25 and 2.50 mM Ca^{2+} respectively.

*, significantly different from respective control period values.

†, significantly different between hearts perfused at different Ca^{2+} concentrations.

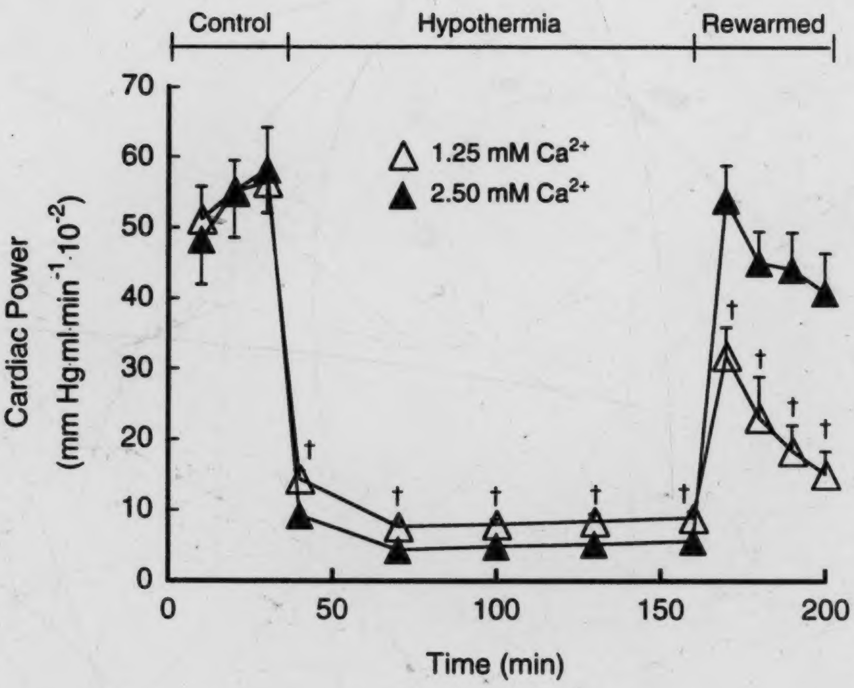


Figure V.1 Changes in cardiac power in rat hearts perfused with either 1.25 or 2.50 mM Ca^{2+}. Values represent mean ± S.E.M. of 14 and 21 rat hearts perfused at 1.25 and 2.50 mM Ca^{2+} respectively.

†, significantly different between hearts perfused with 1.25 and 2.50 mM Ca^{2+} at the same time point.

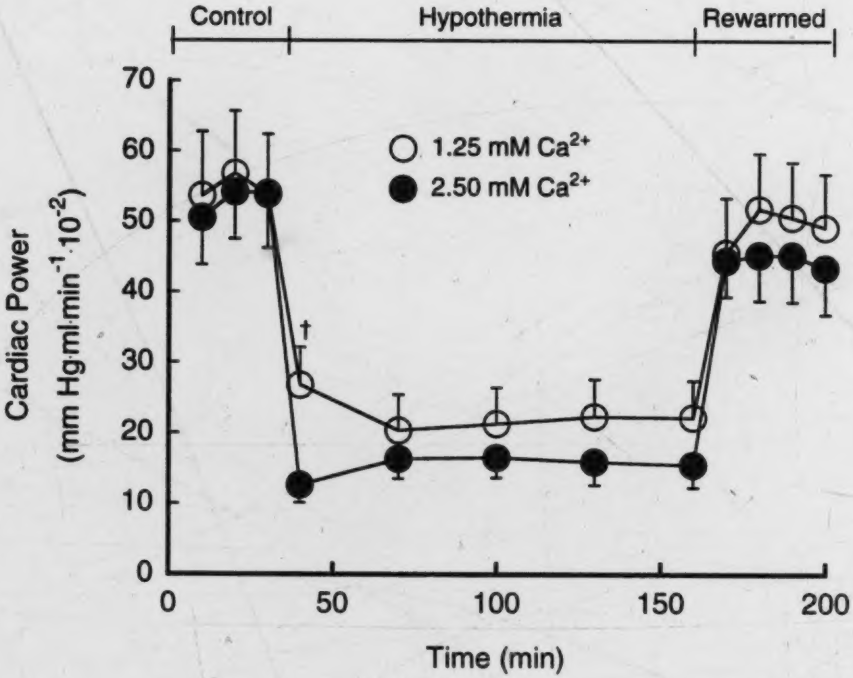


Figure V.2 Changes in cardiac power in ground squirrel hearts perfused with either 1.25 or 2.50 mM Ca^{2+}. Values represent mean ± S.E.M. of 13 and 17 ground squirrel hearts perfused at 1.25 and 2.50 mM Ca^{2+} respectively.
†, significantly different between hearts perfused with 1.25 and 2.50 mM Ca^{2+} at the same time point.

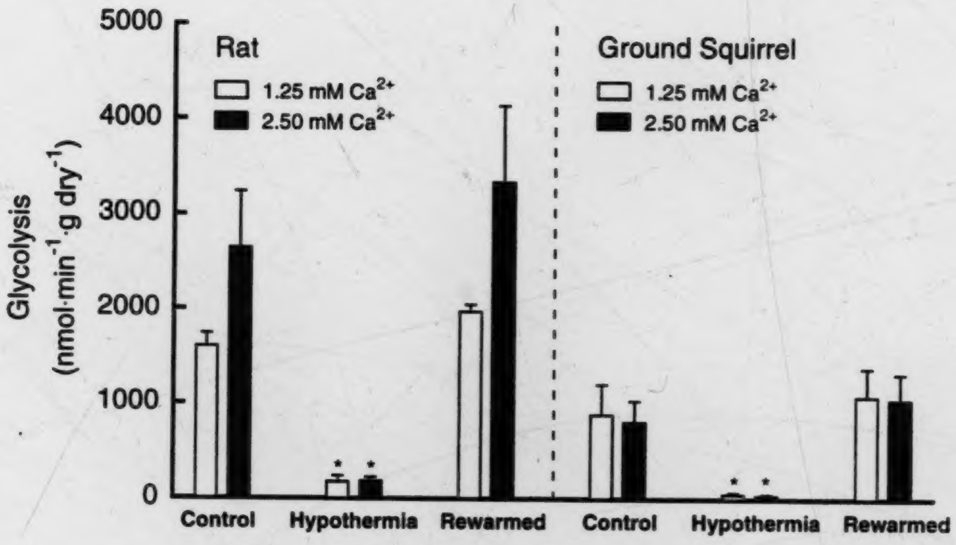


Figure V.3 Steady state rates of glycolysis for rat and ground squirrel hearts during the control, hypothermia and rewarmed periods. Bars represent mean ± S.E.M. of 6 and 9 ground squirrel hearts perfused at 1.25 and 2.50 mM Ca^{2+} and 7 rat hearts perfused at 1.25 and 2.50 mM Ca^{2+} each.

†, significantly different between hearts perfused with 1.25 and 2.50 mM Ca^{2+} during the same period of the protocol.

*, significantly different from respective control period values.

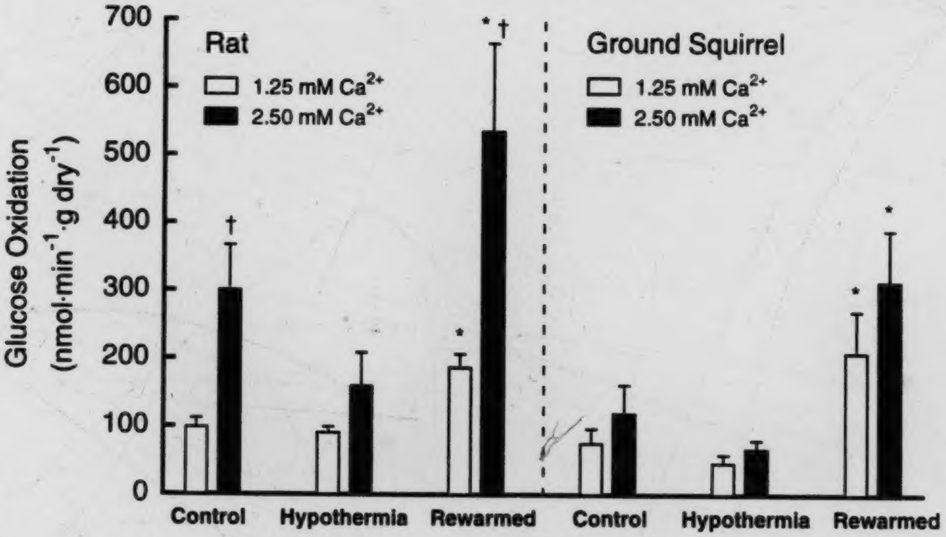


Figure V.4 Steady state rates of glucose oxidation for rat and ground squirrel hearts during the control, hypothermia and rewarmed periods. Bars represent mean ± S.E.M. of 6 and 9 ground squirrel hearts perfused at 1.25 and 2.50 mM Ca^{2+} and 7 rat hearts perfused at 1.25 and 2.50 mM Ca^{2+} each.

†, significantly different between hearts perfused with 1.25 and 2.50 mM Ca^{2+} during the same period of the protocol.

*, significantly different from respective control period values.

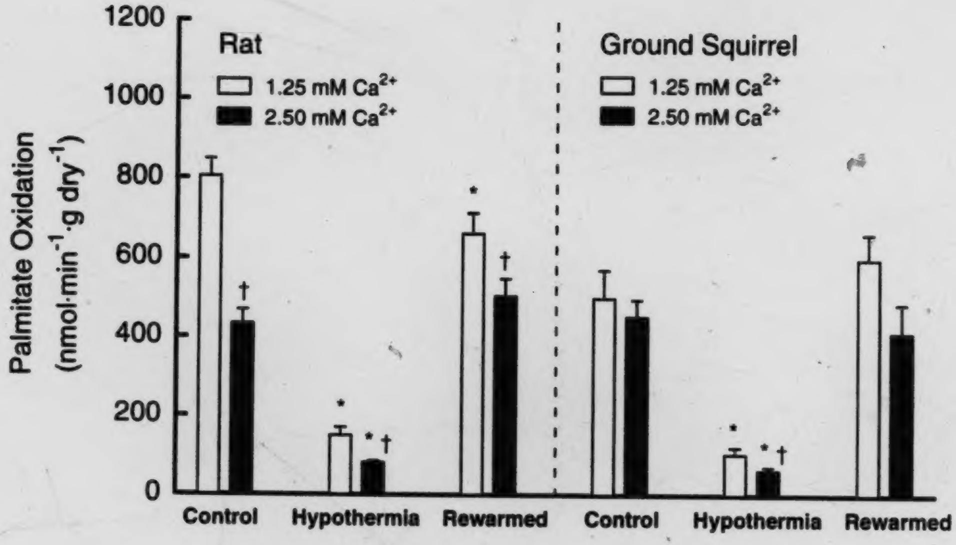


Figure V.5 Steady state rates of palmitate oxidation for rat and ground squirrel hearts during the control, hypothermia and rewarmed periods. Bars represent mean ± S.E.M. of 7 and 8 ground squirrel hearts perfused at 1.25 and 2.50 mM Ca^{2+}, respectively; and 8 and 14 rat hearts perfused at 1.25 and 2.50 mM Ca^{2+}, respectively.

†, significantly different between hearts perfused with 1.25 and 2.50 mM Ca^{2+} during the same experimental period.

*, significantly different from respective control period values.

Figure V.6 Steady state rates of A) glycolysis and B) glucose oxidation for rats and ground squirrels during rewarming (160-170 min). Bars represent mean ± S.E.M. with the 1.25 mM Ca^{2+} treated group being represented by the open bars and the 2.50 mM Ca^{2+} treated group being represented by the closed bars.
†, significantly different between hearts perfused with 1.25 and 2.50 mM Ca^{2+} during the same period of the protocol.

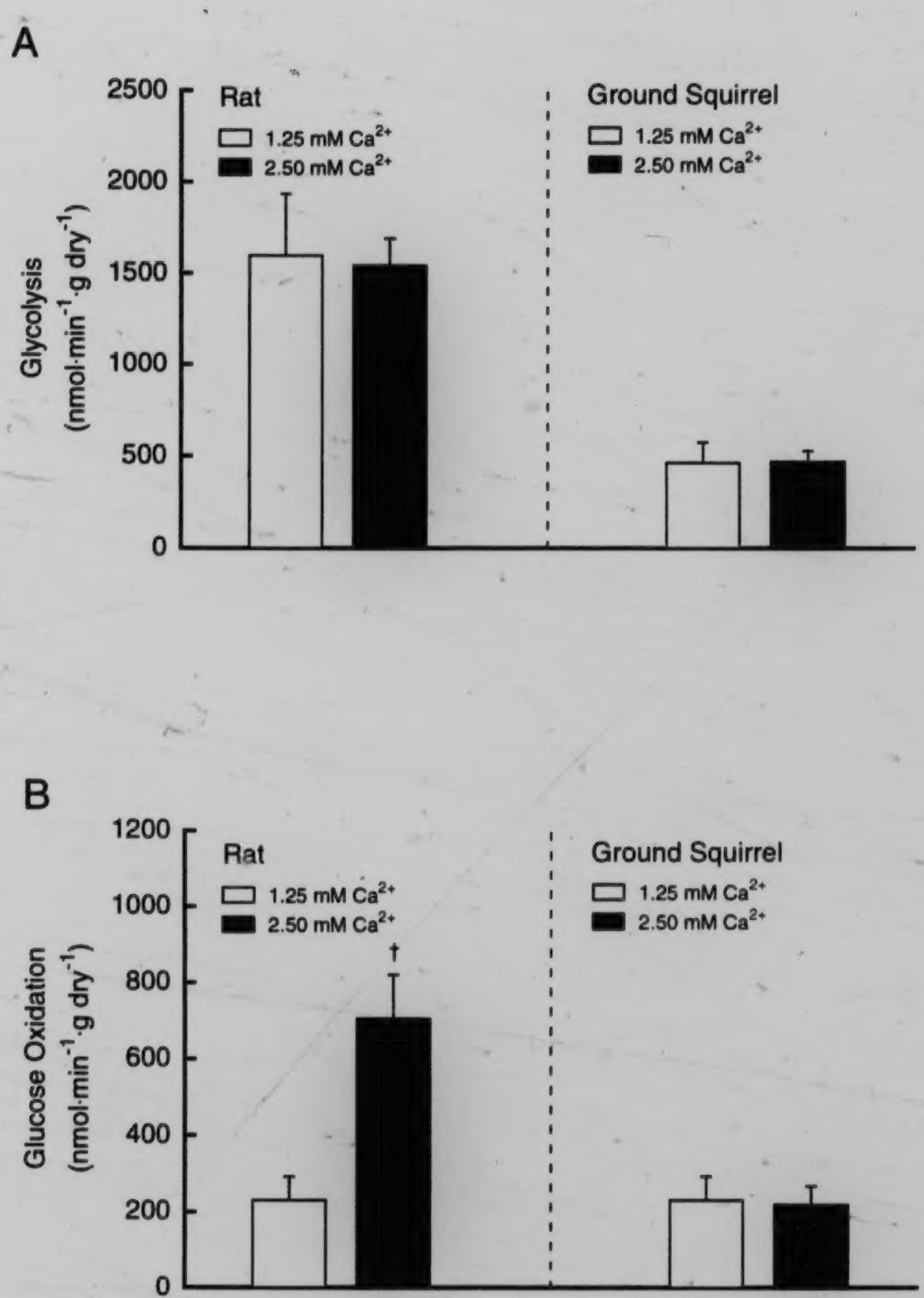

A
Glycolysis
(nmol·min^{-1}·g dry^{-1})
2500
2000
1500
1000
500
0
Rat
1.25 mM Ca^{2+}
2.50 mM Ca^{2+}
Ground Squirrel
1.25 mM Ca^{2+}
2.50 mM Ca^{2+}
B
Glucose Oxidation
(nmol·min^{-1}·g dry^{-1})
1200
1000
800
600
400
200
0
Rat
1.25 mM Ca^{2+}
2.50 mM Ca^{2+}
†
Ground Squirrel
1.25 mM Ca^{2+}
2.50 mM Ca^{2+}

Table V.3

H^+ production in rat and ground squirrel hearts perfused under different Ca^{2+} concentrations.

	H^+ Production	
	1.25 mM Ca^{2+}	2.50 mM Ca^{2+}
Rat	*$(\mu mol \cdot min^{-1} \cdot dry^{-1})$*	
Control	3001 ± 261	4487 ± 905
Hypothermia	219 ± 89*	122 ± 38*
Rewarmed (160-170 min.)	2732 ± 636	1706 ± 345*
Rewarmed (170-200 min.)	3505 ± 1520	4319± 1243
Ground Squirrel	*$(\mu mol \cdot min^{-1} \cdot dry^{-1})$*	
Control	1613 ± 620	851± 246
Hypothermia	34 ± 28*	16 ± 12*
Rewarmed (160-170 min.)	606 ± 221	550 ± 123
Rewarmed (170-200 min.)	1731 ± 563	763 ± 175

Values represent mean ± S.E.M. for 7 rat hearts perfused at 1.25 and 2.5 mM Ca^{2+} each, and 6 and 9 ground squirrel hearts perfused with 1.25 and 2.50 mM Ca^{2+}, respectively.
*, denotes values significantly different from respective control period values.

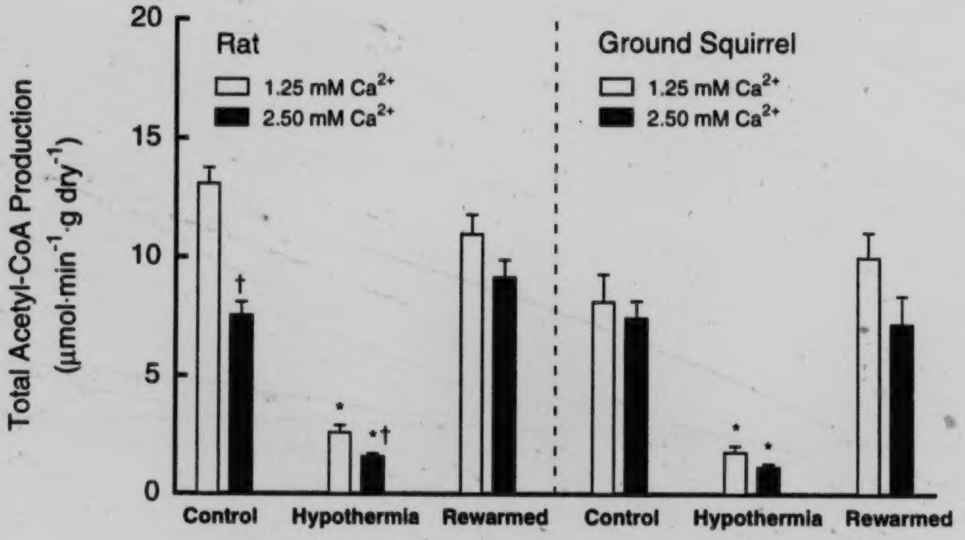


Figure V.7 Total contribution of glucose and palmitate to acetyl-CoA production in mitochondria during the various phases of the perfusion protocol. Bars represent mean ± S.E.M.

†, significantly different between hearts perfused with 1.25 and 2.50 mM Ca^{2+} during the same period of the protocol.

*, significantly different from respective control period values.

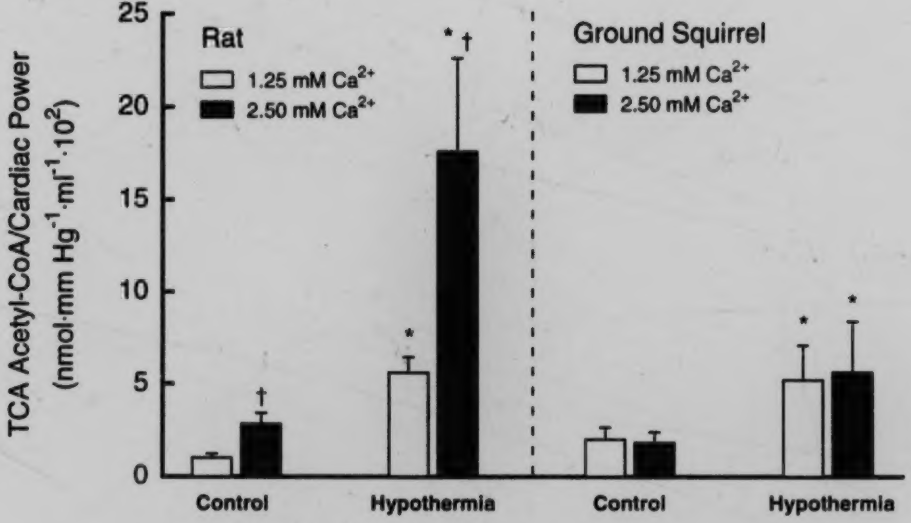
3

Figure V.8 The contribution of A) glucose and B) palmitate to acetyl-CoA production normalized for cardiac power in rat and ground squirrel hearts during the control and hypothermia periods. Bars represent mean ± S.E.M.

†, significantly different between hearts perfused with 1.25 and 2.50 mM Ca^{2+} during the same period of the protocol.

*, significantly different from respective control period values.

A) Glucose



B) Palmitate

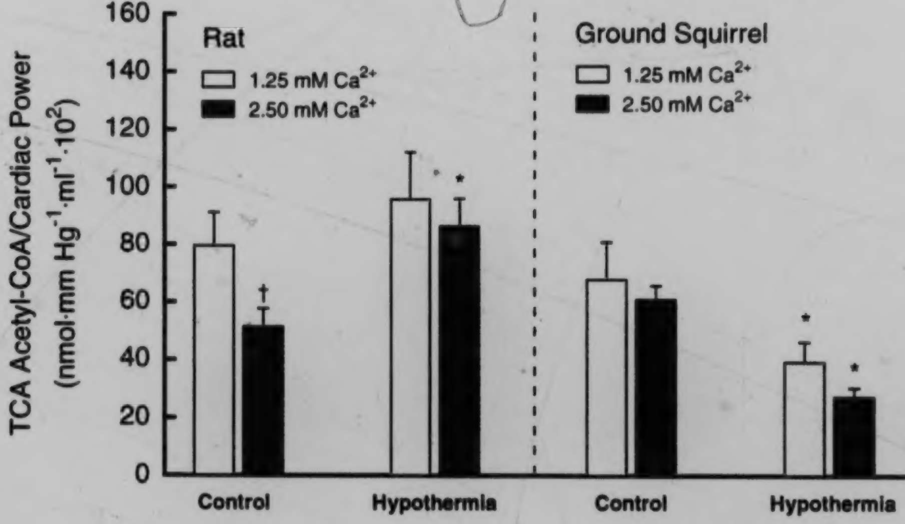


Figure V.9 The relative contribution glycolysis, glucose oxidation and palmitate oxidation to ATP production in the various groups during the A) control, B) hypothermia and C) rewarmed periods of the perfusion protocol. Hatched bars represent the contribution of glycolysis, open bars represent the contribution of glucose oxidation and closed bars represent the contribution of palmitate.

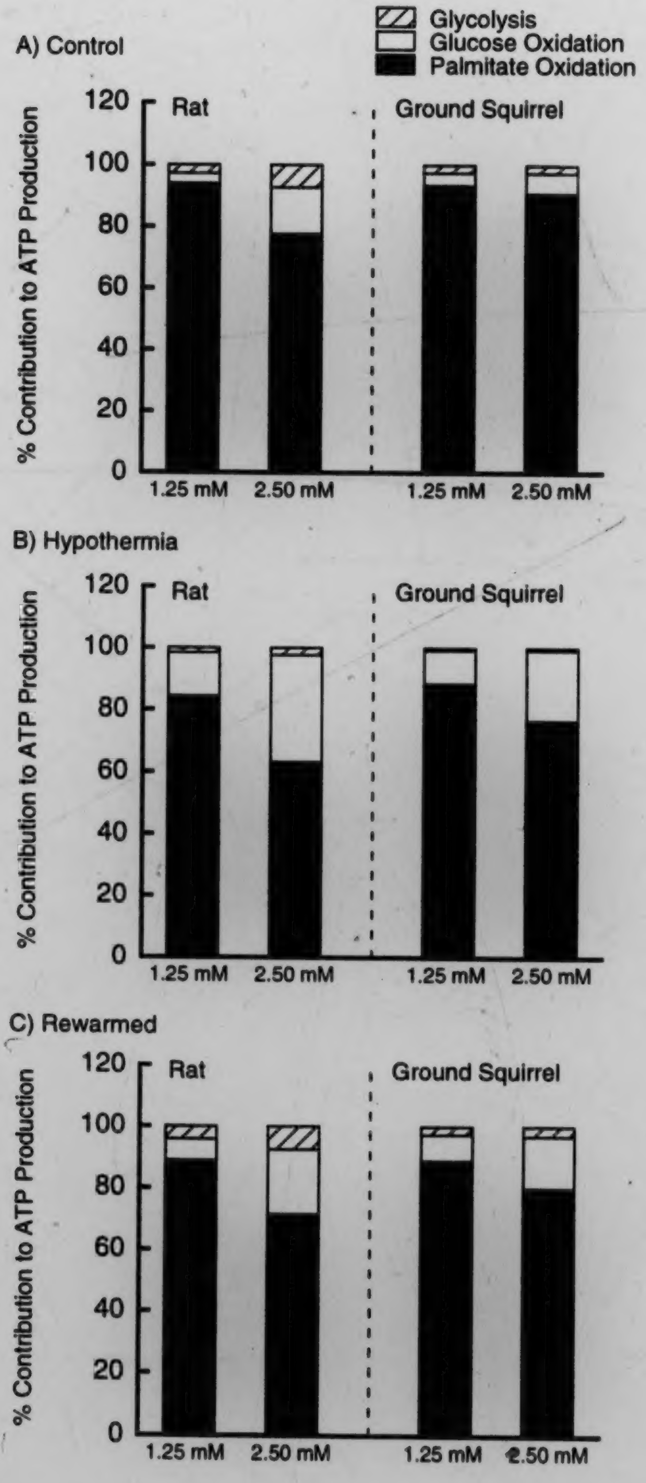

Glycolysis
Glucose Oxidation
Palmitate Oxidation
A) Control
B) Hypothermia
C) Rewarmed
% Contribution to ATP Production
Rat
Ground Squirrel
0
20
40
60
80
100
120
1.25 mM
2.50 mM
1.25 mM
2.50 mM

Table V.4

The efficiency of converting ATP into cardiac power in rat and ground squirrel hearts perfused with different Ca^{2+} concentrations.

	Control	Hypothermia (mm Hg·ml·µmol $ATP^{-1}\cdot 10^{-2}$)	Rewarmed
Rat			
1.25 mM Ca^{2+}	1.79 ± 0.18	1.36 ± 0.16	0.74 ± 0.15*†
2.50 mM Ca^{2+}	2.72 ± 0.37†	1.09 ± 0.15*	1.77 ± 0.25*
Ground Squirrel			
1.25 mM Ca^{2+}	1.77 ± 0.35	3.15 ± 0.83	1.34 ± 0.24
2.50 mM Ca^{2+}	2.02 ± 0.30	3.64 ± 0.57*	1.65 ± 0.34

Values represent mean ± S.E.M.
*, significantly different from respective control period values.
†, significantly different between different Ca^{2+} concentrations within the same species.

Table V.5

Glycogen and triacylglycerol levels from rat and ground squirrel hearts perfused with different Ca^{2+} concentrations.

	Glycogen		Triacylglycerol	
	1.25 mM	2.50 mM	1.25 mM	2.50 mM
Rat	*(μmol glucose ·.g dry^{-1})*		*(μmol ·g dry^{-1})*	
Control	116 ± 13	105 ± 5	20.6 ± 2.5	31.1 ± 2.3
Hypothermia	99 ± 22	120 ± 9	18.7 ± 2.3	27.5 ± 2.3
Rewarmed	122 ± 10	139 ± 11*	22.0 ± 2.5	24.8 ± 3.6
Ground Squirrel	*(μmol glucose ·.dry^{-1})*		*μmol ·g dry^{-1})*	
Control	93 ± 18	100 ± 7	37.0± 5.0	38.9 ± 2.7
Hypothermia	97 ± 10	87 ± 6	38.5 ± 5.8	48.5 ± 3.3
Rewarmed	105 ± 15	101 ± 11	42.8 ± 8.6	38.2 ± 6.0

Values represent mean ± S.E.M. for 6 rat and ground squirrel hearts each, frozen at the end of the control, hypothermia, and rewarmed period.
*, significantly different from respective control period values.

Discussion

The purpose of this study was to determine whether Ca^{2+} affected the performance of rat hearts during hypothermia (i.e. function, efficiency) and whether this ultimately had any affect on the recovery of function following rewarming. As a cold-tolerant species with a good control over intracellular Ca^{2+} under hypothermic conditions, hearts from ground squirrel were included in this study for comparison. Increasing perfusate Ca^{2+} from 1.25 to 2.50 mM did not have an effect on cardiac power output in rat hearts during the control period, confirming previous results suggesting that a change in Ca^{2+} over this concentration range does not induce a positive inotropic effect in isolated working rat hearts (Bielefeld et al., 1983; Schonekess et al., 1995). Under hypothermic conditions the output of cardiac power obtained from rat hearts perfused with 2.50 mM Ca^{2+} was significantly lower than hearts perfused with 1.25 mM Ca^{2+}. Despite this effect, the recovery of cardiac power output was significantly higher in hearts perfused with 2.50 mM Ca^{2+}. As anticipated, function was unaffected by changes in perfusate Ca^{2+} in ground squirrel hearts.

In rat, but not ground squirrel hearts, raising perfusate Ca^{2+} led in general to a significant increase in glucose oxidation and a decrease in palmitate oxidation. The increase in glucose oxidation is likely the result of an increase in mitochondrial Ca^{2+} levels leading to activation of the pyruvate dehydrogenase complex (McCormack and Denton, 1989). The slight increase in glycolysis observed in rat hearts perfused with 2.50 mM Ca^{2+} may reflect a Ca^{2+}-induced stimulation of phosphofructokinase (Leite et al., 1988). The increase in glucose metabolism observed following an increase in perfusate Ca^{2+} concentration is similar to recently published observations (Schonekess et al., 1995). The fact that raising perfusate Ca^{2+} had only minimal effects on energy substrate metabolism in ground squirrel hearts may reflect the better Ca^{2+} regulation in myocytes in this species (Liu et al., 1991, Liu and Wang, 1993), and the inherently higher activity of the sarcoplasmic reticulum (in regulating intracellular Ca^{2+}) observed in ground squirrels in comparison with rats over a wide range of temperatures (Liu et al., 1996).

One of the objectives of this study was to determine if the poor performance of rat hearts during hypothermia, affected the recovery of cardiac function following rewarming. Increasing perfusate Ca^{2+} concentrations led to an increase in the metabolism of glucose and palmitate relative to the level of cardiac power being obtained from these hearts. Despite this decrease in efficiency during hypothermia relative to values obtained during the control period, the recovery of function, and the level of cardiac efficiency was significantly better following rewarming in rat hearts perfused with 2.50 mM Ca^{2+}. This suggests that a decrease in cardiac efficiency during hypothermia does not automatically result in a poorer recovery of function following rewarming. This response differs from that observed under conditions of hypothermic storage involving ischemia, where a rise in intracellular Ca^{2+} is detrimental to the preservation of myocardial function (Stringham et al., 1992; Robinson and Harwood, 1991). The hearts in the present study were not ischemic during hypothermia, yet demonstrated many of the characteristics associated with increased intracellular Ca^{2+} (decreased function, decreased efficiency, increased glucose oxidation), suggesting that the energy wasting effects of Ca^{2+} under hypothermic conditions are not detrimental as long as sufficient energy substrate and O_2 are available.

The main effect of raising perfusate Ca^{2+} on oxidative metabolism was an increase in glucose oxidation and a decrease in palmitate oxidation. However, although raising Ca^{2+} increased glucose oxidation it also increased glycolysis, so that H^+ production did not decrease in these hearts. The pattern of H^+ production as a result of the dissociation of glycolysis and glucose oxidation was similar for rat hearts perfused with different Ca^{2+} concentrations over the course of the perfusion protocol, with the exception of the period of rewarming (160-170 min). During rewarming, glucose oxidation was elevated in rat hearts perfused with 2.50 mM Ca^{2+}, leading to a reduction in H^+ production relative to the control period. Steigen et al (1994) have suggested that the recovery of normal myocardial tissue Ca^{2+} levels following rewarming is beneficial to the recovery of function, and that a return to normal tissue Ca^{2+} levels during rewarming is delayed by the presence of high concentrations of fatty acids. The recovery of normal Ca^{2+} may be delayed by the actions of the Na^+-H^+ and Na^+-Ca^{2+} exchangers if H^+ production is increased during rewarming. Any increase in intracellular H^+ would exchange for Na^+ through the actions of the Na^+-H^+ exchanger, while an increase in intracellular Na^+ could activate the Na^+-Ca^{2+} exchanger leading to an influx of Ca^{2+} during the period of rewarming which would compound the problems of removing the excess Ca^{2+} accumulated during hypothermia. If the recovery of normal intracellular Ca^{2+} during rewarming is dependent upon the reduction of H^+ production, then these observations could explain, in part, why the recovery of function following rewarming was better in the 2.50 mM Ca^{2+} than the 1.25 mM Ca^{2+} group.

The beneficial effect of perfusing rat hearts with 2.50 mM Ca^{2+} may be related to the stimulation of glucose oxidation in these hearts. Under analogous conditions, the stimulation of glucose oxidation has been shown to improve functional recovery following ischemia-reperfusion (Lopaschuk et al., 1993;

Lewandowski and White, 1995, Broderick et al., 1993). Similarly, raising perfusate Ca^{2+} concentrations has also been shown to improve mechanical function in the stunned myocardium (Kupriyanov et al., 1995). However, Ca^{2+} can affect the physiology of the myocardium on a number of different levels ranging from its role in excitation-contraction coupling, to its actions within several second messenger systems. As a result, it is difficult to ascribe any beneficial effect of Ca^{2+}, either as a whole, or in part, to its effect in stimulating glucose oxidation. An increase in glucose oxidation due to direct stimulation of PDC is required to determine what role glucose oxidation plays in mediating the recovery of function following rewarming.

In summary, the recovery of function following rewarming was significantly better in rat hearts perfused with 2.50 mM Ca^{2+} than those perfused with 1.25 mM Ca^{2+}. As expected, hearts from ground squirrel were unaffected by changes in perfusate Ca^{2+} concentration. Although raising perfusate Ca^{2+} decreased function and efficiency in rat hearts during hypothermia, this was not associated with a poorer recovery of function following rewarming. Raising perfusate Ca^{2+} was associated with a higher level of glucose oxidation in rat hearts. This increase in glucose oxidation was not associated with a significant decrease in H^+ production relative to hearts perfused with 1.25 mM Ca^{2+}, however, H^+ production was depressed during rewarming in hearts perfused with 2.50 mM Ca^{2+} due to a faster recovery of glucose oxidation during the period or rewarming. These results suggest that the recovery of function is associated more with the stimulation of glucose oxidation and less with the efficient expenditure of energy under hypothermic conditions. However, due to the extensive effects of Ca^{2+} on cardiac myocyte physiology, the specific stimulation of glucose oxidation is required to determine if improving glucose oxidation is beneficial to functional recovery following rewarming.

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Chapter VI

Dichloroacetate Improves Cardiac Function in Rat Hearts Following Rewarming From Hypothermia

Introduction

The previous studies outlined in Chapters III-V have shown that fatty acids are capable of depressing glucose oxidation under normothermic as well as hypothermic conditions. Furthermore, exposure of hypothermic hearts to fatty acids is associated with a greater depression in function following rewarming (Chapter III, Mjos et al., 1991, Steigen et al., 1994). This effect is analogous to that observed following severe ischemia-reperfusion, where high concentrations of fatty acids also lead to a depression in functional recovery (Lopaschuk et al., 1990, Fralix et al., 1992). Under these conditions an uncoupling of glycolysis and glucose oxidation leads to an increased H^+ production, which is thought to increase intracellular Ca^{2+} through the actions of the Na^+-H^+ and Na^+-Ca^{2+} exchangers (Lopaschuk et al., 1993). Pharmacological treatment aimed at improving glucose oxidation through the use of compounds such as dichloroacetate (DCA), ranolazine or carnitine, have all been shown to be beneficial in improving the recovery of function following ischemia-reperfusion (McVeigh and Lopaschuk, 1990; Broderick et al., 1993, Lewandowski and White, 1995, McCormack et al., 1996). In the previous Chapter (V), an increase in perfusate Ca^{2+} concentration was beneficial in improving the recovery of function following rewarming. This effect was also associated with an increase in glucose oxidation, however, due to the diverse actions of Ca^{2+} within the myocardium a direct relationship between the stimulation of glucose oxidation and the recovery of function following rewarming could not be established. Whether direct stimulation of glucose oxidation in hypothermic hearts is beneficial to the recovery of function following rewarming remains to be directly established.

The entry of carbohydrates into the TCA cycle is controlled by the activity of the pyruvate dehydrogenase complex (PDC) (Patel and Roche, 1991). The activity of PDC is dependent on the rate at which fatty acid oxidation supplies acetyl-CoA to the TCA cycle (Randle et al., 1994; Sugden and Holness, 1994). An increase in the ratio of acetyl-CoA/CoA or NADH/NAD leads to an increase in PDH kinase activity and a decrease in PDH phosphatase activity, resulting in a greater amount of PDC in the phosphorylated inactivated form. Dichloroacetate (DCA), can reverse the inhibitory effects of fatty acid oxidation on PDC activity by inhibiting the action of the PDH kinase (Stacpoole, 1989). Perfusing rat hearts with DCA leads to an increase in PDC activity and an increase in the rate of glucose oxidation (Whitehouse and Randle, 1973; McVeigh and Lopaschuk, 1990; Lewandowski and White, 1995). Administration of DCA has been shown to be beneficial in improving heart function under a number of conditions, including ischemia-reperfusion (McVeigh and Lopaschuk, 1990); congestive heart failure (Bersin et al., 1994), and diabetes (Nichols et al., 1991).

Since our previous studies have indirectly suggested that glucose oxidation is beneficial in the setting of hypothermia-rewarming, we wanted to determine whether the administration of DCA is beneficial in improving functional recovery following hypothermia and rewarming. In the present study, isolated working rat hearts perfused with 1.2 mM palmitate were exposed to DCA prior to hypothermia, or prior to rewarming. We determined if glucose oxidation can be stimulated under these conditions, and if the direct stimulation of glucose oxidation by DCA leads to an improvement in the recovery of function upon rewarming.

Methods

Animals

The animals used in this study were Sprague-Dawley rats.

Heart Perfusions

Isolated working heart perfusions were performed as outlined in Chapter II. The hypothermia-rewarming perfusion protocol was also as described in Chapter II.

Perfusion Conditions

The Krebs'-Henseleit solution used in the experiment contained 1.25 mM free Ca^{2+}. In the "DCA pre-hypothermia" group 1 mM DCA was added to the perfusion solution at the beginning of the experiment. In the "DCA post-hypothermia" group 1 mM DCA was added 5 minutes prior to rewarming. Untreated hearts perfused in the absence of DCA served as control hearts.

O_2 Consumption

O_2 consumption was measured under normothermic as well as hypothermic conditions, as described in Chapter II.

Measurement of Glycolysis, Glucose Oxidation and Palmitate Oxidation

As outlined in Chapter II, glycolysis and glucose oxidation were measured simultaneously in working hearts by perfusing them with [5-^{3}H/U-^{14}C]glucose. Palmitate oxidation was measured in a separate series of hearts perfused with [9,10-^{3}H] palmitate. The contribution of glucose and palmitate to supplying acetyl-CoA to the TCA cycle was calculated by using the stoichiometric ratio of 2 mol acetyl-CoA/mol glucose and 8 mol acetyl-CoA/mol palmitate. Hydrogen ion (H^+) production from the imbalance between glycolysis and glucose oxidation was calculated for each heart by subtracting the rate glucose oxidation from glycolysis and multiplying the resulting number by 2 as outlined by Lopaschuk et al (1993).

Statistical Analysis

A Single Factor Analysis of Variance with a Student-Neuman-Keuls test was used to determine if values were significantly different between control, hypothermia and rewarmed periods within a group, while an unpaired t-test was used when making comparisons between the DCA treated and untreated groups. A value of $p < 0.05$ was considered to be significant. All values presented represent the mean ± S.E.M.

RESULTS

The Effects of DCA on Function

The effects of DCA on heart rate and peak systolic pressure averaged over the different periods of the experiment are shown in Table VI.1, while values for coronary flow, cardiac output and cardiac power are shown in Table VI.2. As no drugs were added to the DCA post-hypothermia group during the control and hypothermia periods, and the values obtained for heart function did not vary significantly from the no addition group for these time periods, these values are omitted from Tables VI.1 and VI.2 for clarity. The addition of 1 mM DCA to the perfusion medium (DCA pre-hypothermia) did not significantly affect any parameters of cardiac function during the initial control period. As expected, hypothermia depressed all indices of heart function in the no addition group. The only significant effect of adding DCA (pre-hypothermia group) was an increase in peak systolic pressure, which resulted in a slightly higher (not significant) output of cardiac power.

Following rewarming, the average level of peak systolic pressure was significantly higher in the DCA pre-hypothermia and the DCA post-hypothermia groups than in the no addition group (Table VI.1). Cardiac output is also significantly higher in the DCA pre-hypothermia group (Table VI.2). As a result, cardiac power output was significantly higher in the DCA pre-hypothermia group than in the no addition group (Table VI.2, Figure VI.1). Although these values were also elevated in the DCA post-hypothermia group, they were not significantly different from untreated hearts, and resulted in a level of cardiac power output which was intermediate to that of the DCA pre-hypothermia group and the no addition group.

Energy Substrate Metabolism

The effect of DCA treatment on substrate metabolism during the normothermic (control, rewarmed) perfusion periods is shown in Table VI.3; while substrate metabolism during the hypothermic period is shown in Figure VI.2. Again, values for the DCA post-hypothermia group are omitted from the control and hypothermia periods for clarity. During the initial control period the addition of DCA to the DCA pre-hypothermia group led to a significant increase in glucose oxidation relative to values from no addition hearts (Table VI.3). The addition of DCA also led to a slight increase in glycolysis and a slight decrease in palmitate oxidation during the control period, although these changes were not significantly different from the no addition hearts. Under hypothermic conditions, DCA led to a significant increase in glucose oxidation and a significant decrease in palmitate oxidation (Figure VI.2). Following rewarming, glucose oxidation in DCA treated hearts was significantly higher than in untreated hearts (Table VI.3).

Glycolysis and Glucose Oxidation During Rewarming

The rates of glycolysis and glucose oxidation during the initial period of rewarming (160-170 min) are shown in Table VI.4. Although presented as a rate, this data does not represent a true steady state rate as with the other time periods due to changes in temperature and cardiac power during this period. Glucose oxidation was significantly higher in both the DCA pre-hypothermia and DCA post-hypothermia groups in comparison to the no addition hearts. Similarly, the highest level of glycolytic activity occurred in the untreated hearts during rewarming; however, this value was not significantly higher than the DCA treated groups.

Contribution of Substrate Metabolism to TCA Acetyl-CoA.

The contribution of glucose and palmitate metabolism to mitochondrial acetyl-CoA supply for the TCA cycle is shown in Table VI.5. During the control period, palmitate supplied the bulk of the acetyl-CoA (> 90%) in all groups regardless of the perfusion conditions. Although the contribution from glucose is lower than that of palmitate, the addition of DCA to the perfusion medium nearly doubled the contribution to total acetyl-CoA production (4.9% to 9.5%). Hypothermia led to a greater depression in palmitate oxidation than glucose oxidation. As a result, the contribution to acetyl-CoA production from glucose was increased in both the DCA treated and no addition hearts during hypothermia. However, the contribution from glucose in the DCA treated hearts was much greater than that of no addition hearts (23.3% vs 7.9%). DCA also increased the contribution of glucose to acetyl-CoA production following rewarming. However, the fact that the percent contribution in the DCA pre-hypothermia group was higher than in the DCA post-hypothermia group was due primarily to the lower overall production of acetyl-CoA in the DCA pre-hypothermia group. Thus, the addition of DCA to the perfusion medium increased the percent contribution of glucose to oxidative metabolism under normothermic as well as hypothermic conditions.

Cardiac Efficiency

To determine what effect DCA has on cardiac efficiency in hypothermic and rewarmed hearts we divided cardiac power by total acetyl-CoA production (Figure VI.3A). Cardiac efficiency was also determined by dividing cardiac power by O_2 consumption measured in a subset of hearts (Figure VI.3B) in order to check the validity of values calculated in Figure VI.3A. Cardiac efficiency during the initial control period did not differ significantly among the groups, regardless of which metabolic parameter was used to calculate cardiac efficiency. During hypothermia, cardiac efficiency decreased in the no addition hearts but not in the hearts treated with DCA pre-hypothermia. As a result, the efficiency of converting energy substrates into cardiac power was significantly higher in the DCA pre-hypothermia group. Following rewarming, the level of efficiency was decreased in all groups relative to the control and hypothermia periods, although the efficiency in the DCA pre-hypothermia group remained significantly higher than the no addition group. In contrast to the DCA pre-hypothermia group, the addition of DCA just prior to rewarming did not result in a significant increase in cardiac efficiency compared to untreated hearts. The effect of DCA on cardiac efficiency was observed in hearts in which cardiac power was divided by either the calculated acetyl-CoA production, or O_2 consumption. This demonstrates that the efficiency calculated using TCA cycle activity accurately reflects the metabolic changes occurring in these hearts as a result of the hypothermia-rewarming protocol.

Energy Substrate Metabolism Normalized for Cardiac Power

The rate of energy substrate metabolism is very dependent on the level of work being performed by the heart. We therefore determined if substrate metabolism normalized for cardiac power output was affected by DCA treatment. Figure VI.4 shows glucose oxidation and palmitate oxidation for the control and hypothermic periods normalized for cardiac power. Glucose oxidation normalized for cardiac power increased significantly in all groups during hypothermia. In contrast, palmitate oxidation normalized for cardiac power was unchanged by DCA treatment (values decreased slightly in the DCA pre-hypothermia group). This suggests that the effects of DCA during hypothermia can be directly attributed to DCA's effect on glucose oxidation. Following rewarming, both glucose oxidation and palmitate oxidation normalized for cardiac power increases in all groups (data not shown), suggesting that both substrates contribute to the reduction in cardiac efficiency observed in all groups during the rewarmed period.

Hydrogen Ion Production

H^+ production due to the imbalance between glycolysis and glucose oxidation is shown in Table VI.6. H^+ production was similar in all groups during the initial control and hypothermia periods, despite the decrease in H^+ production during hypothermia. During rewarming (160-170 min), H^+ production was significantly lower in both groups of DCA treated hearts when compared to the no addition hearts. This was due to the higher level of glucose oxidation observed in DCA treated hearts during the period of rewarming. Following rewarming (170-200 min) the rate of H^+ production was similar among all experimental groups (Table VI.4). Although H^+ production was reduced during rewarming in both DCA treated groups, only the DCA pre-hypothermia group showed a significant improvement in the recovery of function following rewarming.

Table VI.1

The effects of dichloroacetate on heart rate and peak systolic pressure in hypothermic and rewarmed hearts.

	Heart Rate (beats·min^{-1})	PSP (mm Hg)
Control		
No Addition	236 ± 7	113 ± 5
DCA Pre-hypothermia	218 ± 8	120 ± 3
Hypothermia		
No Addition	27 ± 3	71 ± 5
DCA Pre-hypothermia	31 ± 2	86 ± 3†
Rewarmed		
No Addition	220 ± 18	70 ± 11
DCA Pre-hypothermia	229 ± 11	97 ± 4†
DCA Post-hypothermia	257 ± 8	92 ± 5†

Values are the mean ± S.E.M. for 14 no addition hearts, 13 hearts treated with DCA pre-hypothermia, and 16 hearts treated with DCA post-hypothermia. PSP, peak systolic pressure

†, denotes values which are significantly different from no addition hearts.

Table VI.2

The effects of dichloroacetate on coronary flow, cardiac output, and cardiac power in hypothermic and rewarmed hearts.

	Coronary Flow $(ml \cdot min^{-1})$	Cardiac Output $(ml \cdot min^{-1})$	Cardiac Power $(mm\,Hg \cdot ml \cdot min^{-1} \cdot 10^{-2})$
Control			
No Addition	21.7 ± 2.1	42.9 ± 3.8	47 ± 5
DCA Pre-hypothermia	24.9 ± 1.6	39.7 ± 3.1	48 ± 4
Hypothermia			
No Addition	6.1 ± 0.7	6.3 ± 0.8	5 ± 1
DCA Pre-hypothermia	7.9 ± 0.7	10.5 ± 2.3	9 ± 2
Rewarmed			
No Addition	14.9 ± 2.9	16.6 ± 3.4	14 ± 3
DCA Pre-hypothermia	18.9 ± 2.8	25.0 ± 3.5†	26 ± 4†
DCA Post-hypothermia	18.6 ± 1.7	20.1 ± 2.6	19 ± 3

Values are the mean ± S.E.M. for 14 no addition hearts, 13 hearts treated with DCA pre hypothermia, and 16 hearts treated with DCA post hypothermia.
†, denotes values which are significantly different from the no addition hearts.

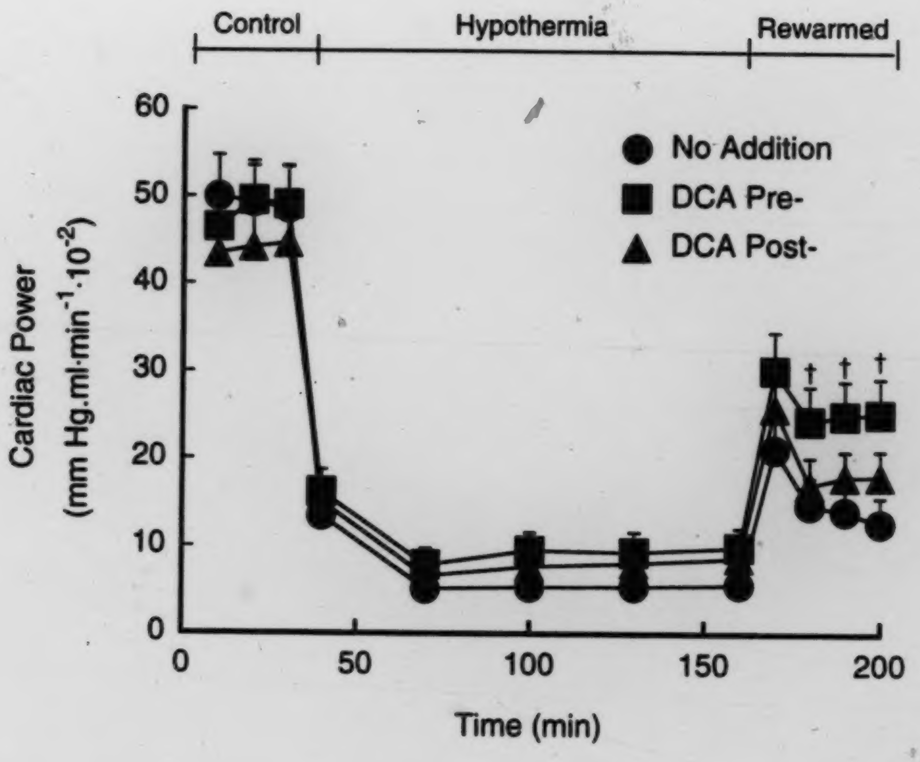


Figure VI.1 Cardiac power in hearts perfused with 11 mM glucose and 1.2 mM palmitate in the presence or absence of 1 mM dichloroacetate (DCA). Values represent mean ± S.E.M. for 14 no addition hearts, 13 heart with DCA pre-hypothermia and 16 hearts with DCA post hypothermia.
†, denotes values significantly different from the no addition hearts.

Table VI.3

Substrate oxidation during normothermic perfusion in rat hearts perfused with or without dichloroacetate.

	Glycolysis	Glucose Oxidation *(nmol·g dry^{-1}·min^{-1})*	Palmitate Oxidation
Control			
No Addition	974 ± 177	171 ± 26	831 ± 62
DCA Pre-hypothermia	1287 ± 132	364 ± 42†	697 ± 67
Rewarmed			
No Addition	906 ± 136	234 ± 34	745 ± 54
DCA Pre-hypothermia	1197 ± 75	540 ± 65†	609 ± 161
DCA Post-hypothermia	1128 ± 93	575 ± 48†	937 ± 128

Values are the mean ± S.E.M for 8 no addition hearts, 7 hearts treated with DCA pre-hypothermia and 9 hearts treated with DCA post-hypothermia. †,denotes values significantly different from the no addition hearts.

Figure VI.2 Steady state rates of glycolysis (A), glucose oxidation (B) and palmitate oxidation (C) during hypothermia in hearts perfused with or without 1 mM dichloroacetate (DCA). Values represent mean ± S.E.M. for 8 no addition hearts and 7 DCA pre-hypothermia hearts.

†, denotes values significantly different from the no addition hearts during the same phase of the protocol.

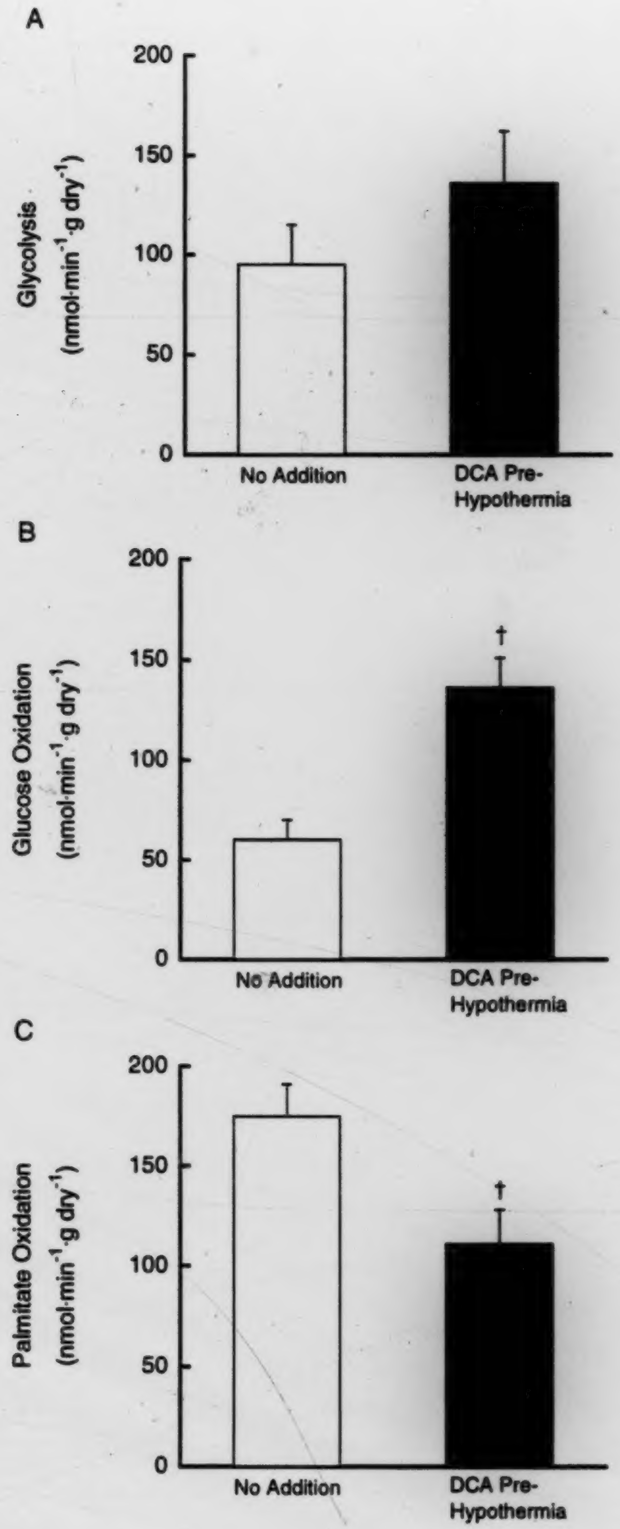

A
Glycolysis
(nmol·min^{-1}·g dry^{-1})
200
150
100
50
0
No Addition
DCA Pre-Hypothermia
B
Glucose Oxidation
(nmol·min^{-1}·g dry^{-1})
200
150
100
50
0
†
No Addition
DCA Pre-Hypothermia
C
Palmitate Oxidation
(nmol·min^{-1}·g dry^{-1})
200
150
100
50
0
†
No Addition
DCA Pre-Hypothermia

Table VI.4

Glycolysis and glucose oxidation during the initial period of rewarming (160 - 170 min).

	Glycolysis	Glucose Oxidation
	(nmol·g dry^{-1}·min^{-1})	
No Addition	1446 ± 136	297 ± 41
DCA Pre-hypothermia	1093 ± 141	555 ± 91†
DCA Post-hypothermia	1217 ± 155	643 ± 88†

Values are the mean ± S.E.M for 8 no addition hearts, 7 hearts treated with dichloroacetate (DCA) pre-hypothermia and 9 hearts treated with DCA post-hypothermia.
†, denotes values significantly different from the no addition hearts.

Table VI.5

Steady state acetyl-CoA production in hypothermic and rewarmed hearts treated with or without dichloroacetate .

	Glucose Oxidation	Palmitate Oxidation	Total Production
		(μmol Acetyl-CoA·g dry^{-1}·min^{-1})	
Control			
No Addition	0.34 ± 0.04 (4.9%)	6.65 ± 0.50 (95.1%)	6.99 ± 0.51
DCA Pre-hypothermia	0.73 ± 0.08 (9.5%)	4.99 ± 0.55 (90.5%)	7.72 ± 0.56
Hypothermia			
No Addition	0.12 ± 0.02 (7.9%)	1.40 ± 0.13 (92.1%)	1.52 ± 0.13
DCA Pre-hypothermia	0.27 ± 0.03 (23.3%)	0.89 ± 0.13 (76.7%)	1.16 ± 0.14
Rewarmed			
No Addition	0.47 ± 0.07 (7.3%)	5.96 ± 0.43 (92.7%)	6.43 ± 0.44
DCA Pre-hypothermia	1.08 ± 0.13 (18.2%)	4.87 ± 0.49 (81.8%)	5.95 ± 0.57
DCA Post-hypothermia	1.15 ± 0.09 (13.3%)	7.50 ± 1.03 (86.7%)	8.65 ± 1.04

Acetyl-CoA production rates were calculated from the data presented in Table VI.3 and Figure VI.2. The values in brackets represent the percent contribution towards total acetyl-CoA production.

Figure VI.3 Cardiac efficiency during the various phases of the perfusion protocol of hearts perfused with 11 mM glucose + 1.2 mM palmitate in the presence or absence of 1 mM dichloroacetate (DCA). A) Cardiac efficiency calculated from values for total Acetyl-CoA production as shown in Table VI.5. B) Cardiac efficiency calculated independently from O_2 consumption measurements. Values represent mean ± S.E.M. for A) 14 no addition hearts, 13 DCA pre-hypothermia hearts and 16 DCA post-hypothermia perfused hearts; and B) 6 no addition hearts, 9 DCA pre-hypothermia hearts and 7 DCA post-hypothermia perfused hearts.

†, denotes values significantly different from the no addition hearts during the same phase of the protocol.

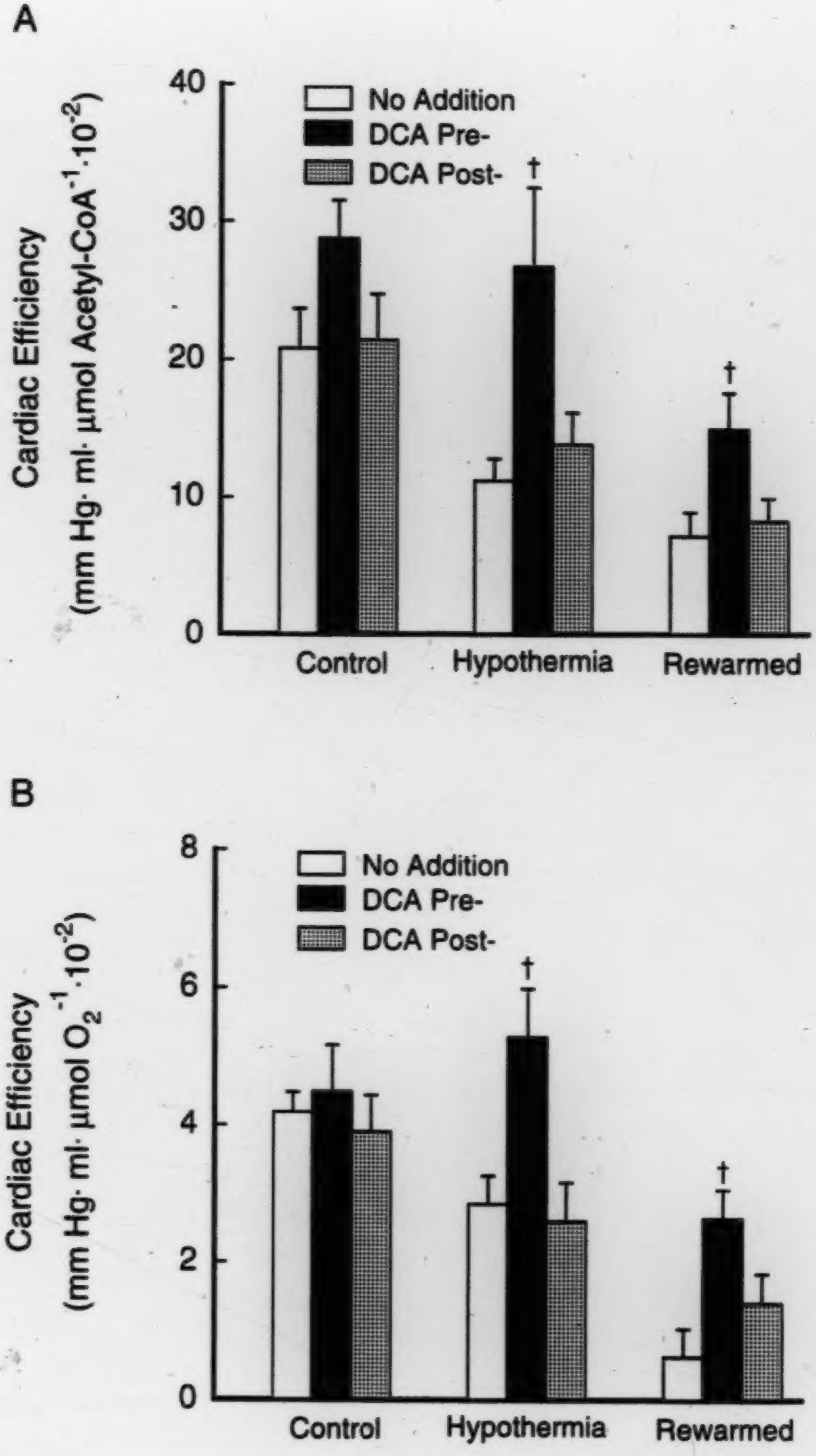
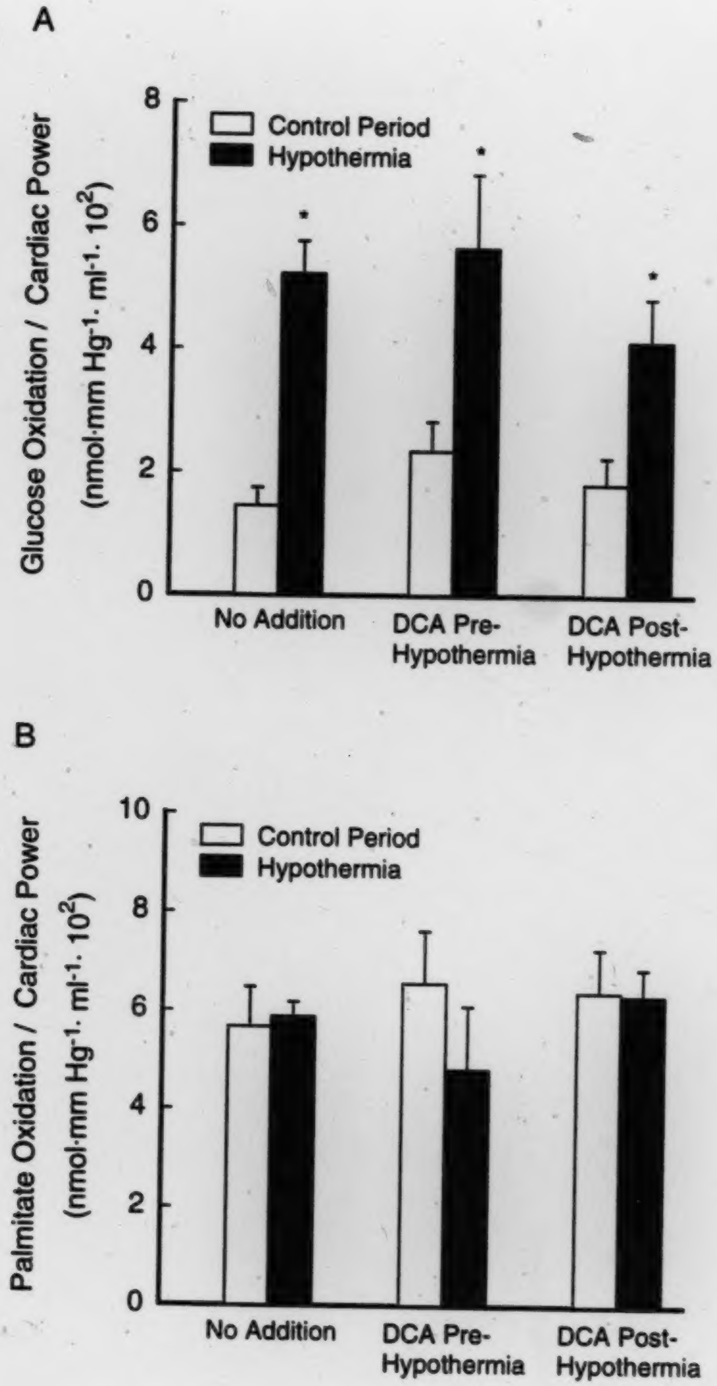

A
Cardiac Efficiency
(mm Hg· ml· µmol Acetyl-CoA^{-1}·10^{-2})
0
10
20
30
40
No Addition
DCA Pre-
DCA Post-
†
†
Control
Hypothermia
Rewarmed
B
Cardiac Efficiency
(mm Hg· ml· µmol O$_2$$^{-1}$·10^{-2})
0
2
4
6
8
No Addition
DCA Pre-
DCA Post-
†
†
Control
Hypothermia
Rewarmed

Figure VI.4 Substrate oxidation normalized for cardiac work for glucose oxidation (A) and palmitate oxidation (B) during the initial control and hypothermia periods for hearts perfused with 11 mM glucose and 1.2 mM palmitate. Values represent mean ± S.E.M. for A) 8 no addition hearts, 7 dichloroacetate (DCA) pre-hypothermia hearts and 9 DCA post hypothermia hearts; and B) 6 no addition hearts, 6 DCA pre-hypothermia hearts and 7 DCA post-hypothermia perfused hearts.
*, denotes values significantly different from initial control period values.


A
Glucose Oxidation / Cardiac Power
(nmol·mm Hg^{-1}· ml^{-1}· 10^{2})
8
6
4
2
0
Control Period
Hypothermia
*
*
*
No Addition
DCA Pre-Hypothermia
DCA Post-Hypothermia
B
Palmitate Oxidation / Cardiac Power
(nmol·mm Hg^{-1}· ml^{-1}· 10^{2})
10
8
6
4
2
0
Control Period
Hypothermia
No Addition
DCA Pre-Hypothermia
DCA Post-Hypothermia

TableVI.6

The effect of hypothermia and rewarming on H+ production from the imbalance between glycolysis and glucose oxidation.

	Control	Hypothermia	Rewarmed	
	(0-30 min)	(40-160 min)	(160-170 min)	(170-200 min)
		(nmol $H^{+}\cdot min^{-1}\cdot g\ dry^{-1}$)		
No Addition	1643 ± 416	93 ± 23	2297 ± 317	1273 ± 329
DCA Pre-hypothermia	1618 ± 298	56 ± 25	1107 ± 305†	1193 ± 117
DCA Post-hypothermia	1660 ± 317	53 ± 45	1246 ± 353†	1157 ± 232

Values represent mean ± S.E.M for 8 no addition hearts, 7 dichloroacetate (DCA) pre-hypothermia treated hearts, 9 DCA post-hypothermia treated hearts.

†, denotes values which are significantly different from the no addition hearts.

Discussion

This study examined whether the direct stimulation of glucose oxidation by DCA was beneficial to the recovery of function in rewarmed hearts following hypothermia. If DCA (1 mM) was present prior to hypothermia, the recovery of cardiac power was significantly higher than that obtained from no addition hearts. In contrast, the addition of DCA just prior to rewarming did not lead to a significant improvement in the recovery of heart function. As a result, hypothermia-rewarming differs from ischemia-reperfusion, where DCA is only beneficial if added during reperfusion, and confers no protective effect if added prior to ischemia (McVeigh and Lopaschuk, 1990). While the detrimental effect of DCA added prior to ischemia is related to an increase in H^+ production during ischemia, the beneficial effect of DCA added prior to hypothermia was not due to a reduction in H^+ production during hypothermia.

The addition of DCA to the perfusion medium led to a significant increase in glucose oxidation, which was associated with a significant decrease in palmitate oxidation during the hypothermic period. DCA is thought to decrease palmitate oxidation through the actions of increased malonyl-CoA production from ACC and inhibition of CPT 1 (Saddik et al., 1993). Our results suggest that DCA is also effective in inhibiting palmitate oxidation under hypothermic conditions. As a result, DCA increased the contribution of glucose to overall oxidative metabolism under both normothermic and hypothermic conditions. This was accompanied by an increase in cardiac efficiency during hypothermia in the DCA pre-hypothermia group. This suggests that increasing glucose oxidation is a potential approach to decreasing hypothermic injury.

The ability of DCA to improve the efficiency and contractile function of the heart under pathological conditions such as congestive heart failure and ischemia-reperfusion has been noted previously (Bersin et al., 1994, Lewendowski and White, 1995). Although DCA had similar effects on cardiac efficiency and contractile function in this study, it was necessary that DCA was present during the actual hypothermia period to obtain the beneficial effects during rewarming. Addition of DCA just prior to rewarming did not lead to a significant improvement in cardiac efficiency relative to untreated hearts.

In ischemic hearts, the beneficial effect of DCA is thought to be due to a decrease in H^+ production as the result of an improvement in the coupling of glycolysis to glucose oxidation during reperfusion (Lopaschuk et al., 1993). In the present study, DCA did not significantly alter H^+ production from glucose metabolism with the exception of the rewarming period (160-170 min), where DCA led to a significant reduction in H^+ production in both the DCA pre-hypothermia group and the DCA post-hypothermia group relative to no addition hearts. This occurred as the result of a stimulation of glucose oxidation relative to glycolysis during this time period. However, although glucose oxidation was stimulated, and H^+ production was depressed, in hearts treated with DCA post-hypothermia, this failed to significantly improve the recovery of function during rewarming. This suggests that a reduction in H^+ production during rewarming is not the primary factor determining the recovery of function following rewarming. These results also suggest that the stimulation of glucose oxidation during hypothermia is more beneficial to recovery than the stimulation of glucose oxidation during rewarming alone. However, the possibility that DCA added during hypothermia failed to stimulate glucose oxidation during the early phase of rewarming or whether DCA requires higher temperatures to inhibit PDH kinase cannot be excluded by this study.

In the previous Chapter (V), an increase in perfusate Ca^{2+} was associated with an improvement in functional recovery following rewarming, and a stimulation of glucose oxidation. However, due to the myriad of effects that increasing perfusate Ca^{2+} can have on the heart, we were unable to establish a direct link between increasing glucose oxidation and improving functional recovery. By directly stimulating glucose oxidation with DCA, we were able to show that improving glucose oxidation is beneficial to the recovery of function following rewarming; however, the beneficial effects are not related to a reduction in H^+ production following rewarming. This suggests that the beneficial actions of DCA during hypothermia-rewarming differs from those during ischemia reperfusion. Clearly, the role of DCA in improving cardiac efficiency during rewarming cannot account for this effect, as cardiac efficiency during hypothermia was dissociated from functional recovery by the results presented in the previous Chapter. As a result, the stimulation of glucose oxidation must work by some other mechanism. The stimulation of PDC makes the myocyte more responsive to changes in metabolic demand (From et al., 1990), leading to an improvement in cytosolic phosphorylation potential. Energy substrates which stimulate PDC activity such as pyruvate have been shown to be beneficial in improving Ca^{2+} handling by the sarcoplasmic reticulum (Mallet and Bunger, 1994). As previous studies have shown that sarcoplasmic reticulum activity is depressed following hypothermia (Liu et al., 1993) it is conceivable that an improvement in glucose oxidation improves excitation-contraction coupling in the rewarmed heart.

In summary, the addition of DCA prior to hypothermia leads to a significant improvement in functional recovery following rewarming. DCA is capable of stimulating glucose oxidation under both normothermic and hypothermic conditions. This supports the data presented in Chapter V that suggested an increase in glucose oxidation can benefit the hypothermic heart. The exact mechanism by which an increase in glucose oxidation benefits the hypothermic heart remains to be determined.

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Chapter VII

Acetyl-CoA Carboxylase Control of Fatty Acid Oxidation in Hearts from Hibernating Richardson's Ground Squirrels

The previous Chapters have shown that fatty acids are capable of depressing glucose metabolism under normothermic and hypothermic conditions, and that a stimulation of glucose oxidation under hypothermic conditions is beneficial to the recovery of function following rewarming. In rat hearts the stimulation of glucose oxidation with DCA is associated with a decrease in fatty acid oxidation. In ground squirrel hearts, an increase in glucose oxidation normalized for cardiac power output during hypothermia is associated with a decreased contribution from fatty acids. If a balance between the oxidation of glucose and fatty acids plays a role in affecting function during and following hypothermia, then ACC activity, which is important in the control of CPT 1, should play a role in determining the balance of carbohydrate and fatty acid oxidation under hypothermic conditions. As hibernation is thought to lead to an increased reliance on fatty acids as a source of energy, ACC activity should play a role in regulating substrate metabolism during this physiological state.

In hibernating species, entry into the hibernating phase is accompanied by an increased storage of body fat. During hibernation, these animals exhibit a respiratory quotient (CO_2/O_2) of around 0.7, indicating that these animals rely primarily on fatty acids as a source of energy (South and House, 1967). In support of this, Yacoe (1983) found an increased preference for the oxidation of fatty acids in homogenates of the pectoralis muscle of hibernating brown bats compared to non-hibernating bats. In the heart, an increased reliance on fatty acid oxidation has been suggested in hibernation, based on an increase in long-chain acylcarnitine levels and an increase in the number of lipid droplets observed in electron micrographs (Burlington et al., 1972; Burlington and Shug, 1980). Additional indirect evidence for an increased reliance of hibernating hearts on fatty acids was provided by Brooks and Story (1992), who found that the activities of phosphofructokinase (PFK) and the pyruvate dehydrogenase complex (PDC), key enzymes for glucose utilization, are down regulated in heart tissue from hibernating ground squirrels. Despite this evidence, no direct measurement of fatty acid or glucose oxidation has been made in hearts from hibernating or non-hibernating ground squirrels. Furthermore, while control of glucose metabolism has been examined in hibernating species, little is known about the control of fatty acid oxidation in the hearts of hibernators.

The activity of carnitine palmitoyltransferase-1 (CPT-1), which catalyzes the conversion of acyl-CoA into acyl-carnitine, controls the entry of fatty acid into the mitochondria. This enzyme is inhibited by malonyl-CoA, the product of acetyl-CoA carboxylase (ACC) (McGarry et al., 1989). Although the inhibition of CPT-1 by malonyl-CoA is important in preventing the simultaneous synthesis and oxidation of fatty acids in tissues capable of also synthesizing fatty acids (e.g. liver, adipose tissue), isoforms of ACC have recently been identified in tissues not involved in fatty acid synthesis (e.g. heart and skeletal muscle) (Bianchi et al., 1990, Thampy, 1989). In rat hearts, ACC primarily functions to regulate fatty acid oxidation (Saddik et al., 1993, Awan and Saggerson, 1993), since cardiac CPT 1 is extremely sensitive to inhibition by malonyl-CoA (McGarry et al, 1983, Cook, 1984). Under hypothermic conditions, the role of ACC in regulating fatty acids may be increased, as previous studies (on liver mitochondria) have shown that CPT 1 is more sensitive to inhibition by malonyl-CoA at lower temperatures (Zammit et al., 1984; Zammit and Corstorphine, 1985; Kolodziej and Zammit, 1990; Kashfi and Cook, 1995). This suggests that the activity of ACC during hibernation may play an important role in regulating fatty acid oxidation in the heart at low temperatures.

Whether the ACC control of fatty acid oxidation is altered in hearts of hibernating species, especially at low temperatures, has not been determined. In this study we directly measured glucose and fatty acid oxidation rates in hearts obtained from hibernating and non-hibernating Richardson's ground squirrels. Isolated working hearts were perfused at both 37 and 5° C, to determine if the contribution of fatty acids to oxidative metabolism increases during hibernation, and whether changes in ACC activity follow any changes in fatty acid oxidation in these hearts.

Methods

The isolated working heart preparation was used as described in Chapter II. Glucose and fatty acid oxidation were measured simultaneously using [U-^{14}C]glucose and [9,10-^{3}H]palmitate and determining the production of $^{14}CO_2$ and $^{3}H_2O$ as outlined in Chapter II. The hypothermia protocol consisted of 30 minutes of perfusion at 37° C followed by cooling to 5° C and perfusion for 60 minutes. In both the non-hibernating and the hibernating group, 4 hearts were frozen at the end of the 30 minute euthermic period and 5 hearts were frozen at the end of hypothermia. ACC was isolated using the PEG 6000 precipitation method and its activity was measured at 37 and 5° C. ACC isoform distribution was determined by image analysis from streptavidin blots of each heart used in the experiment.

Hearts were harvested from hibernating ground squirrels which had been in hibernation for a period ranging from 2 weeks to 3 months. The animals were rewarmed to 37°C prior to the collection of the hearts.

Differences between hibernating and non-hibernating ground squirrels were determined using an unpaired t-test, with significance being set at $p < 0.05$.

Results

Cardiac Function.

Body mass of the animals and the functional parameters of the hearts 37 and 5° C are shown in Table VII.1. Due to fat accumulation, ground squirrels in the hibernating phase had a significantly larger body mass than non-hibernating ground squirrels. In general, the functional parameters of isolated working hearts did not differ significantly between hearts from hibernating and non-hibernating animals when measured at 37° C, with the exception of heart rate, which was significantly higher in the non-hibernating group. In both groups, cooling the heart to 5° C led to a decrease in all functional parameters, with no significant difference being observed between hearts from hibernating and non-hibernating animals.

Energy Substrate Metabolism

The average steady state rates for glucose oxidation and palmitate oxidation in hearts perfused at 37 and 5° C are shown in Table VII.2. Glucose oxidation rate for the non-hibernating group was significantly higher than that observed in the hibernating group at both 37 and 5° C. In contrast, palmitate oxidation rate was not significantly different between the groups at either temperature. It should be noted that both groups of hearts were perfused with similar concentrations of fatty acids, even though circulating fatty acid levels are higher in hibernating squirrels vs non-hibernating squirrels (Galster and Morrison, 1966).

Reducing heart temperature to 5° C resulted in a greater inhibition of palmitate oxidation than glucose oxidation. However, while glucose oxidation was depressed to a similar extent in both groups (≈ 45% of 37° C values) regardless of starting levels, palmitate oxidation rates decreased to 5% of the values seen at 37° C in the non-hibernating group, but to only to 15% of 37° C values in the hibernating group.

Since glucose and palmitate oxidation rates were measured simultaneously in all hearts, the percent contribution of palmitate and glucose oxidation to the overall TCA cycle acetyl-CoA supply could be calculated at different temperatures (Table VII.2). During the 37° C perfusion, the contribution of palmitate as a source of TCA cycle acetyl CoA was slightly, but significantly, higher in the hibernating group. Cooling to 5° C led to an approximately 90% decrease in TCA cycle activity, which roughly matches the decrease in cardiac power observed in these two states (Table VII.1).

As glucose oxidation was less affected by temperature than palmitate oxidation, the contribution of glucose to oxidative metabolism increased in both groups at 5° C (Figure VII.1A). However, since palmitate oxidation was less affected by the decrease in temperature in hearts from hibernators, the contribution of palmitate oxidation to TCA cycle activity was significantly higher when compared to the non-hibernators (80% vs 49% at 5° C, respectively) (Figure VII.1B). Thus, relative to the differences in the metabolism of the individual substrates, hibernating ground squirrels rely more heavily on fatty acid oxidation as a source of energy at low temperatures.

Acetyl-CoA Carboxylase

To determine if the differences in fatty acid oxidation were paralleled by changes in ACC activity, ACC was measured in hearts frozen at the end of the 37 and 5° C periods. As shown in Figure VII.2, ACC activity was significantly higher in non-hibernating ground squirrels hearts perfused at 37° C. Cooling of hearts to 5° C led to a reduction in enzyme activity to ≈3% of 37° C values for each group. However, even at these low rates, ACC activity remained lower in the hearts from hibernating animals when compared with non-hibernating animals. In the absence of citrate the rate of ^{14}C fixation into malonyl-CoA was lower in both groups at 37° C (0.79 $nmol\cdot min^{-1}\cdot mg\ protein^{-1}$ and 0.72 $nmol\cdot min^{-1}\cdot mg\ protein^{-1}$, non-hibernating vs. hibernating, respectively) and at 5° C (0.017 $nmol\cdot min^{-1}\cdot mg\ protein^{-1}$ and 0.025 $nmol\cdot min^{-1}\cdot mg\ protein^{-1}$, non-hibernating vs. hibernating, respectively). This suggests that ACC from both groups is sensitive to regulation by citrate.

Tissue levels of acetyl-CoA and malonyl-CoA in hearts frozen at the end of the 37 and 5° C perfusion were also measured in these hearts. Acetyl-CoA levels were not significantly different in the non-hibernating or hibernating groups at either 37° C (19.8 ± 1.2 vs. 18.4 ± 2.5 nmol/g dry, respectively) or 5° C (12.5 ± 4.7 vs. 7.8 ± 2.1 nmol/g dry, respectively), although acetyl-CoA levels did decrease in both groups upon cooling. Malonyl-CoA levels were also not significantly different in the non-hibernating or hibernating groups at 37° C (3.6 ± 0.2 vs. 3.6 ± 0.3 nmol/g dry, respectively). However, malonyl-CoA levels were significantly higher in hearts in the non-hibernating group when compared with the hibernating group for hearts frozen at the end of hypothermia (5° C) (4.3 ± 0.2 vs. 3.2 ± 0.3 nmol/g dry, respectively).

ACC Expression

To determine if the ACC activity between hearts from hibernating and non-hibernating animals could be explained by differences in ACC expression, ACC isoforms were isolated and quantified from both groups. As has been shown in rat hearts (Saddik et al., 1993), ACC is present in two isoforms in both hibernating and non-hibernating ground squirrels, with the 280 kDa isoform being the major isoform, and the 265 kDa isoform being the minor isoform (Figure VII.3A). Analysis of isoform distribution within all hearts from each group revealed that the minor 265 kDa isoform was similar between the groups, while the major 280 kDa isoform was significantly lower in the hibernating group, (i.e. 24% lower than the non-hibernating group) (Figure VII.3B).

ACC Activity and Fatty Acid Oxidation

To determine if differences in ACC activity correlated with fatty acid oxidation rates in hibernating and non-hibernating animals, the percent contribution of fatty acids to oxidative metabolism was plotted against the ACC activity for the individual hearts (Figure VII.4). At both 37 and 5° C, the percent contribution of palmitate oxidation to oxidative metabolism was negatively correlated to the level of ACC activity. The correlation coefficient was -0.66 at 37° C and -0.82 at 5° C The higher correlation at 5° C was due to the larger range of the contribution of fatty acids to oxidative metabolism. This suggests that ACC activity plays a role in mediating the preferential utilization of fatty acids as a substrate, with the lower expression and activity of ACC in hibernating ground squirrel hearts resulting in an increased reliance on fatty acids as a source of energy.

Table VII.1

Body mass and isolated working heart function in hibernating and non-hibernating ground squirrels

Perfusion Temperature	Body Mass (grams)	Heart Rate (beats·min^{-1})	PSP (mm Hg)	Cardiac Output (ml·min^{-1})	Cardiac Power (mm Hg·ml·min^{-1}·10^{-2})
37° C					
Non-Hibernating	350 ± 17	353 ± 9	105 ± 10	52.4 ± 7.3	57.1 ± 9.5
Hibernating	628 ± 34†	291 ± 21†	105 ± 9	57.3 ± 7.6	63.8 ± 11.5
5° C					
Non-Hibernating	375 ± 11[a]	20 ± 2	65 ± 5	8.5 ± 1.6	5.7 ± 1.3
Hibernating	635 ± 59[a]†	20 ± 3	70 ± 5	10.8 ± 3.3	7.1 ± 1.8

Values are the mean ± S.E.M. for 9 hearts in each group perfused at 37° C and 5 hearts in each group perfused at 5° C.

[a], note 5 of the hearts perfused at 37° C were subsequently perfused at 5° C.

†, denotes values which are significantly different between groups.

Table VII.2

Glucose oxidation and palmitate oxidation rates and their contribution to TCA cycle acetyl-CoA supply in hearts from hibernating and non-hibernating ground squirrels.

Perfusion Temperature	Glucose Oxidation (nmol min^{-1} g dry^{-1})	Palmitate Oxidation (nmol min^{-1} g dry^{-1})	Overall TCA cycle activity (nmol acetyl-CoA min^{-1} g dry^{-1})
37°C			
Non-Hibernating	339 ± 58	805 ± 69	5774 ± 498
Hibernating	172 ± 37†	641 ± 93	4597 ± 673
5°C			
Non-Hibernating	158 ± 35	50 ± 12	693 ± 115
Hibernating	55 ± 18†	75 ± 12	656 ± 86

Values are the mean ± S.E.M. for 9 ground squirrel in each group perfused at 37° C and 5 ground squirrel in each group perfused at 5°C.
†, denotes values which are significantly different between groups.

Figure VII.1 Percent contribution of glucose oxidation (A) and palmitate oxidation (B) at 37 and 5° C in hearts from non-hibernating and hibernating animals. Bars represent mean ± S.E.M. for n = 9 hearts at 37° C and n = 5 hearts measured at 5°C.

†, denotes values significantly different between groups at the same temperature.

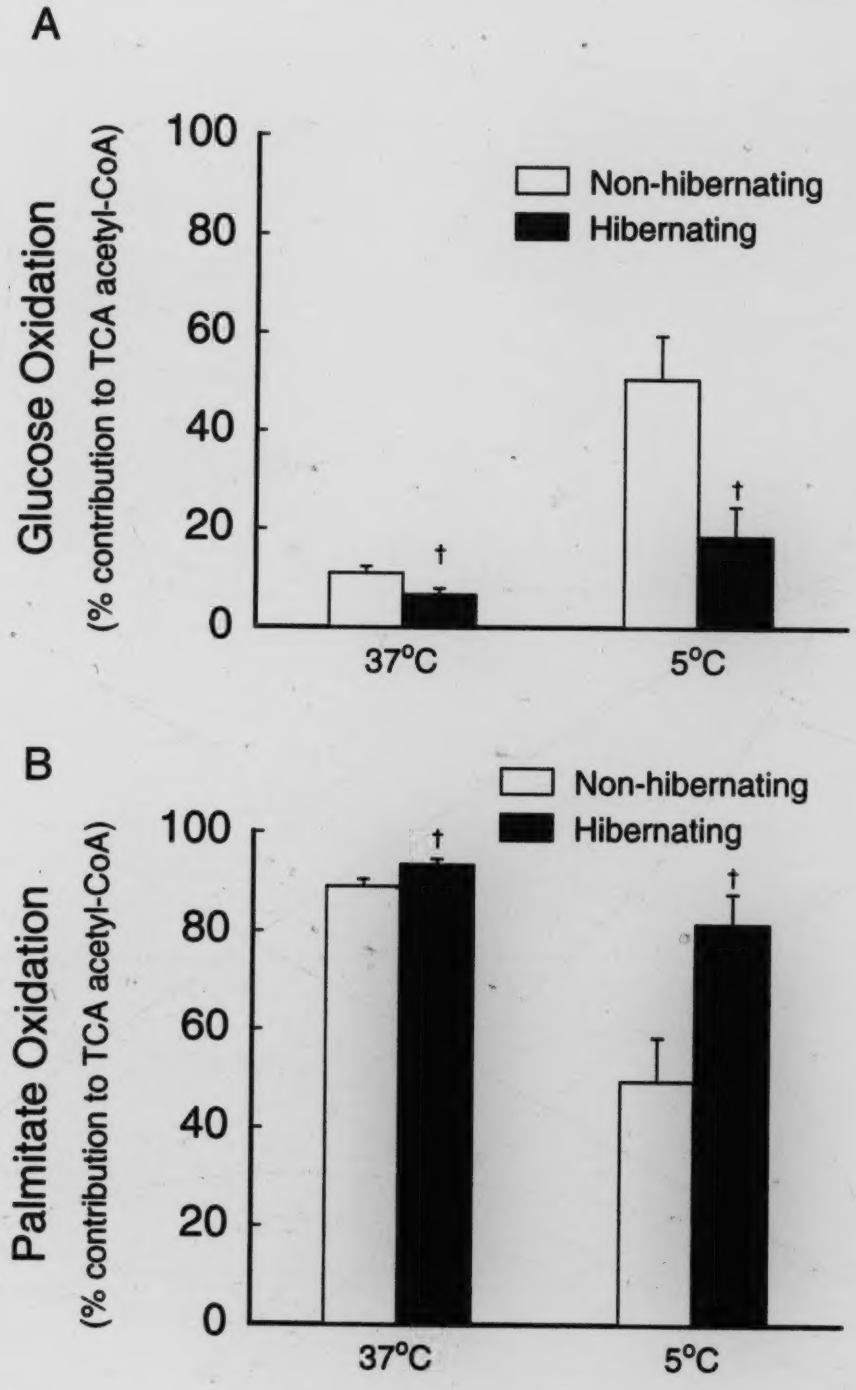

A
Glucose Oxidation
(% contribution to TCA acetyl-CoA)
100
80
60
40
20
0
Non-hibernating
Hibernating
†
†
37°C
5°C
B
Palmitate Oxidation
(% contribution to TCA acetyl-CoA)
Non-hibernating
Hibernating
100
80
60
40
20
0
†
†
37°C
5°C

Figure VII.2 ACC activity measured in the presence of 10 mM citrate at 37° C (A) and 5° C (B). Bars represent mean ± S.E.M. for n = 9 samples for each group.

†, denotes values significantly different between groups at the same temperature.

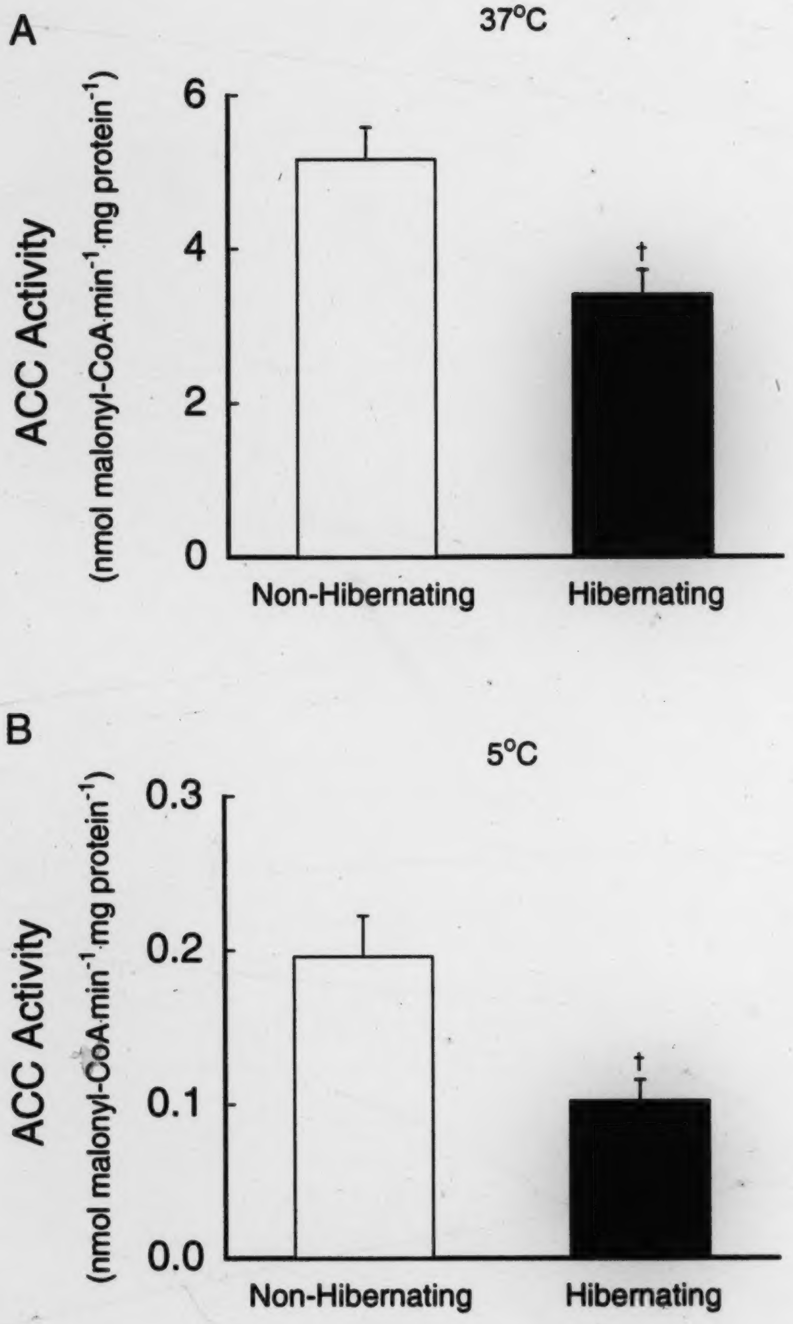

A
37°C
ACC Activity
(nmol malonyl-CoA·min-1·mg protein-1)
6
4
2
0
†
Non-Hibernating
Hibernating
B
5°C
ACC Activity
(nmol malonyl-CoA·min-1·mg protein-1)
0.3
0.2
0.1
0.0
†
Non-Hibernating
Hibernating

Figure VII.3 Streptavidin blot of the ACC fraction isolated from the hearts of non-hibernating and hibernating ground squirrels (A) and the relative amounts of the 265 and 280 kDa ACC isoform measured by determining band density using image analysis of the streptavidin blot (B).

In Figure 3A the upper band represents the 280 kDa isoform while the band below it represents the 265 kDa isoform. The amount of protein added to each lane is shown at the bottom of the Figure. In Figure 3B the bars represent mean ± S.E.M. for n = 9 samples for each group.
†, denotes values significantly different between groups at the same temperature.

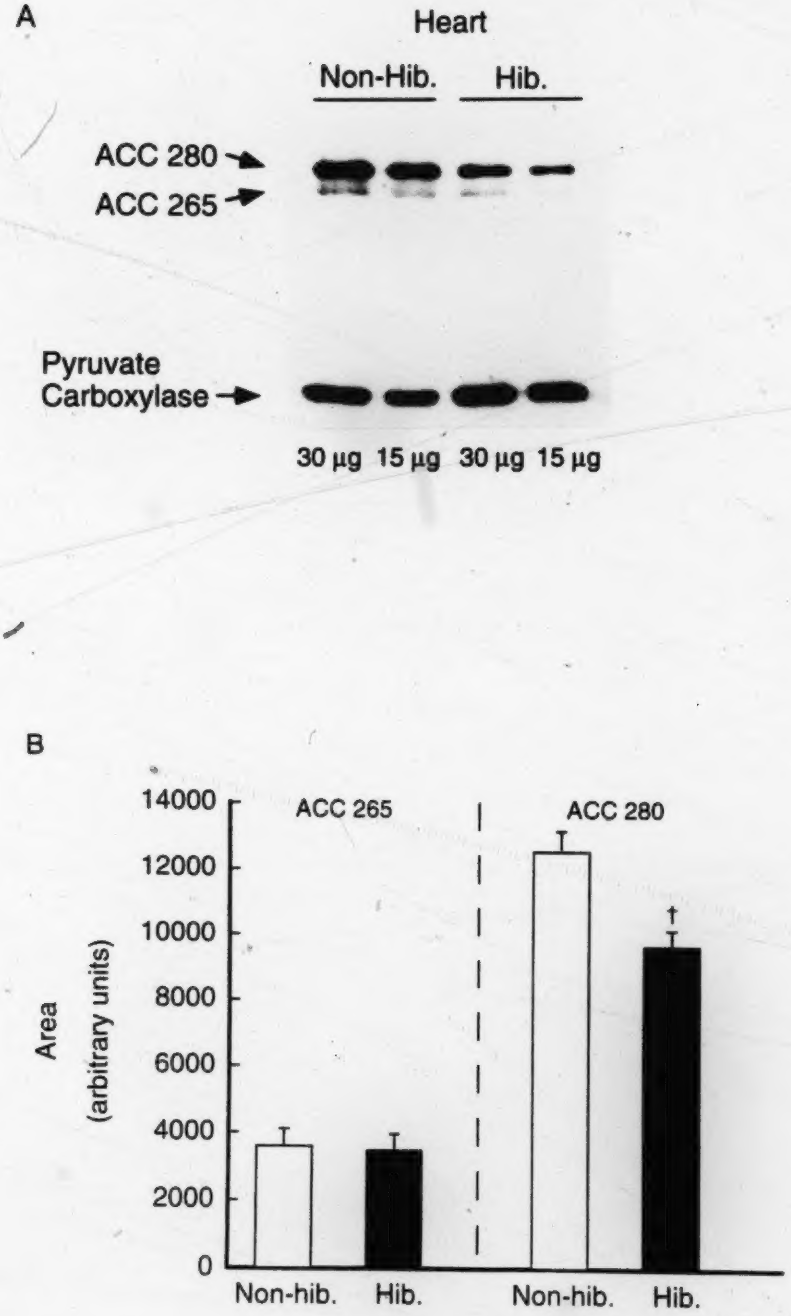
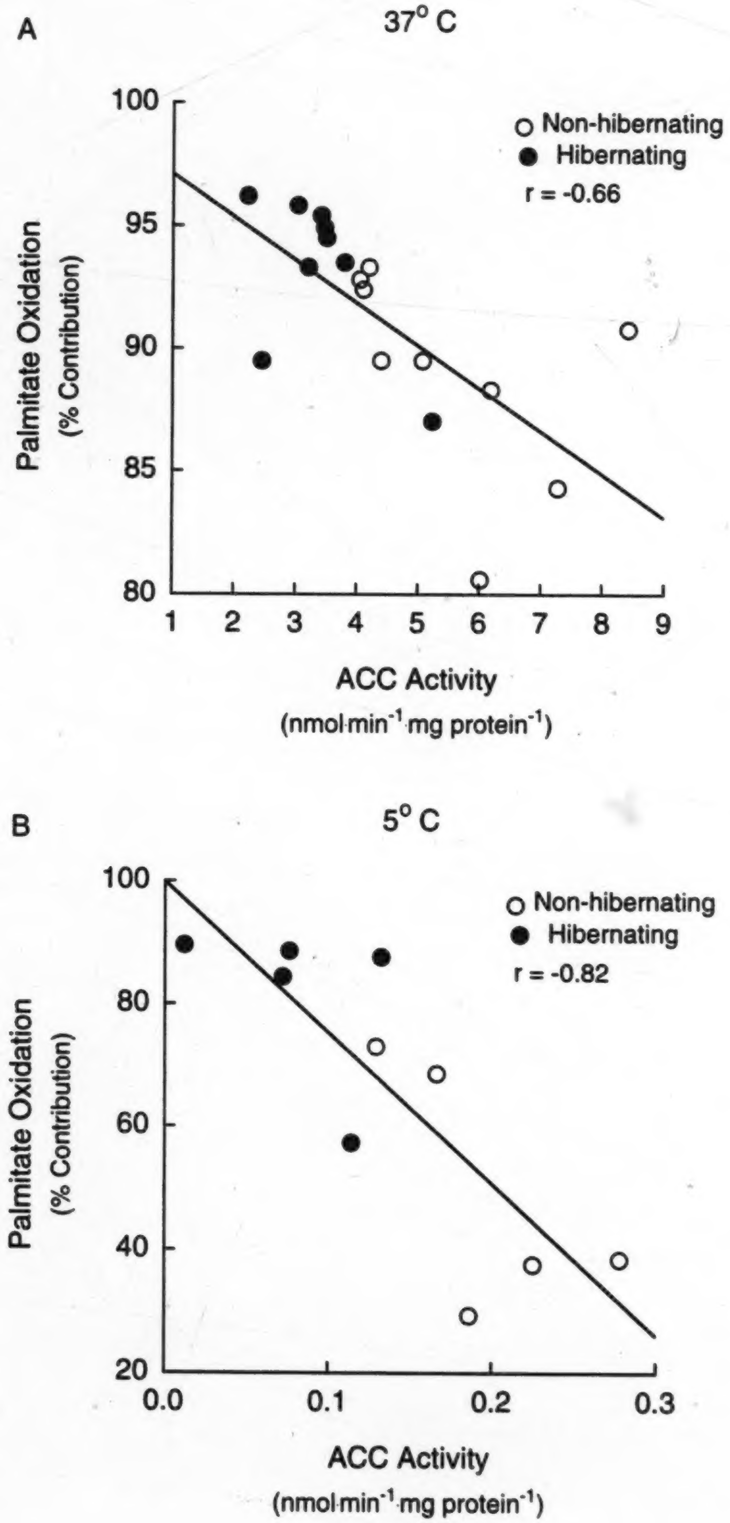

A
Heart
Non-Hib.
Hib.
ACC 280
ACC 265
Pyruvate
Carboxylase
30 µg
15 µg
30 µg
15 µg
B
14000
12000
10000
8000
6000
4000
2000
0
Area
(arbitrary units)
ACC 265
ACC 280
†
Non-hib.
Hib.
Non-hib.
Hib.

Figure VII.4 Correlation between the percent contribution of palmitate to oxidative metabolism and ACC activity measures for individual hearts at 37° C (A) and 5° C (B).


A
37° C
100
95
90
85
80
Palmitate Oxidation
(% Contribution)
○ Non-hibernating
● Hibernating
r = -0.66
1
2
3
4
5
6
7
8
9
ACC Activity
(nmol·min^{-1}·mg protein^{-1})
B
5° C
100
80
60
40
20
Palmitate Oxidation
(% Contribution)
○ Non-hibernating
● Hibernating
r = -0.82
0.0
0.1
0.2
0.3
ACC Activity
(nmol·min^{-1}·mg protein^{-1})

Discussion

This study examined whether hibernating ground squirrel were more reliant on fatty acids as a source of energy in the hibernating state, and whether the activity of ACC played any role in mediating this effect. As expected, animals in the hibernating state had a significantly higher body weight than those in the non-hibernating state. Apart from a slower heart rate in the hibernating group at 37° C, functional parameters did not differ significantly between the two groups. The level of glucose oxidation obtained from hearts in the hibernating group was lower than that obtained from non-hibernating hearts, however, hypothermia depressed glucose oxidation in both groups to a similar extent. In contrast, the level of palmitate oxidation was not significantly different between groups. However, cooling to 5° C did not lead to a greater level of depression of palmitate oxidation in the non-hibernating group than in the hibernating group. As a result, the percent contribution of fatty acid to overall oxidative metabolism is significantly higher in hibernating (81.5%) than non-hibernating hearts (49.4%) at 5° C. This suggests that fatty acid oxidation in cold-tolerant species does not present a problem for normal cardiac function under hypothermic and rewarmed conditions.

Analysis of ACC content revealed that the level of the major 280 kDa isoform was significantly lower (≈ 24% lower) in the hibernating than in the non-hibernating group. In contrast, the level of the minor 265 kDa isoform was similar between the groups. The activity of ACC was also significantly lower in the hibernating than the non-hibernating group. This effect was noted at both 37 and 5° C, indicating that ACC activity between the groups is not differentially affected by cooling. It should be noted that the ACC activity measured using the ^{14}C-bicarbonate fixation method is not specific for the action of ACC and may reflect the activity of other carboxylases such as propionyl-CoA carboxylase. As a result it is possible that some of the ACC activity presented in this study occurred as a result of the activity of propionyl-CoA carboxylase.

Malonyl-CoA level measured in hearts frozen at the end of the 5° C perfusion period revealed that the malonyl-CoA level was significantly higher in non-hibernating hearts than hibernating hearts. The large difference in the contribution of fatty acids to energy metabolism between the groups at 5° C could reflect the greater sensitivity of CPT 1 to malonyl-CoA inhibition at low temperatures (Zammit et al., 1984; Zammit and Corstorphine, 1985; Kolodziej and Zammit, 1990; Kashfi and Cook, 1995). The percent contribution of fatty acids to oxidative metabolism was found to be negatively correlated to the activity of ACC measured for individual hearts at 37 and 5°C. This suggests that ACC activity does play a role in regulating the contribution of fatty acids to oxidative metabolism under normothermic and hypothermic conditions.

In summary, hearts from hibernating animals were shown to be more dependent on fatty acid oxidation as a source of energy at both 37 and 5° C. This increased reliance on fatty acids was associated with a decrease in ACC 280 kDa expression and activity in hearts from hibernating ground squirrels. A good correlation ($r = -0.82$ at 5° C) was obtained between the activity of ACC (measured in the presence of citrate) and the percent contribution of fatty acids to oxidative metabolism in hearts of hibernating and non-hibernating ground squirrels.

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Zammit, V. A., and Corstorphine, C. G. (1985) Effects of incubation at physiological temperature on the concentyration-dependence of [2-^{14}C]malonyl-CoA binding to rat liver mitochondria. Biochem. J. 231:343-347.

Zammit, V. A.,Corstorphine, C. G., and Gray, S. R. (1984) Changes in the ability of malonyl-CoA to inhibit carnitine palmitoyltranserase 1 activity and to bind to rat liver mitochondria during incubation in vivo. Biochem. J. 222:335-342.

Chapter VIII

General Discussion

Although widely used during surgery as a means of reducing metabolic demand during ischemia, the effect of hypothermia itself on heart function and energy metabolism has received little attention. Hypothermia, combined with a high K^+ cardioplegia, is used to reduce the metabolic demand of the heart during surgery in order to avoid post-operative impairment of heart function. However, recent studies have suggested that exposure of the heart to low temperature cardioplegia is less favorable than warm cardioplegia, leading to increased creatine kinase release and a reduction in the recovery of heart function (Yau et al. 1993; Mauney and Kron, 1995). Similarly, whole animal hypothermia followed by rewarming leads to a reduction in left ventricular force development and cardiac output which can eventually result in circulatory collapse (Tvieta et al., 1996, 1994). Thus hypothermia is capable of causing detrimental effects on heart function.

Although hypothermia is often viewed as being favorable in delaying the onset of ischemic injury by preserving energy stores (ATP, creatine phosphate), both hypothermia and ischemia share a number of similarities in their effects on heart function. Both lead to a disruption of ion homeostasis, resulting in increased intracellular Na^+ and Ca^{2+} concentrations (Martineau-Knerr and Lieberman, 1993; Tani and Neely, 1989). Both can also lead to a depression in sarcoplasmic reticulum function (Krause et al., 1989; Liu et al. 1994, Labow et al., 1993), the development of arrhythmias (Johansson, 1996; Oliver and Opie, 1994), myocardial stunning and a reduction in cardiac efficiency (DeMaison and Grynberg, 1994; Tveita et al., 1994). Both hypothermia and ischemia can also complement each other (i.e. detrimental effects are additive) in the damage they cause to myocyte integrity and heart function (Ting et al., 1994; Ferrera et al., 1994, Navas et al., 1992). Finally, the recovery of function in hearts subjected to both ischemia-reperfusion and hypothermia-rewarming can be depressed by exposing the hearts to high concentrations of fatty acids (Lopaschuk et al., 1994a; Fralix et al., 1992; Mjos et al., 1991, Steigen et al., 1994). Although the effect of fatty acids in contributing to cardiac dysfunction has been examined for a number of pathological conditions (diabetes, ischemia-reperfusion, etc.), the effect of fatty acids on function and metabolism during hypothermia-rewarming has not been previously examined prior to the initiation of this Ph.D. research project. Studying the effect of fatty acids during hypothermia is clinically relevant as plasma fatty acid concentrations are elevated during heart surgery (Storstein et al., 1979; Svensson et al., 1990; Lopaschuk et al., 1994b), such that reperfusion of a hypothermic heart following surgery will expose the heart to high concentrations of fatty acids. Similarly, the use of the patients blood during cold blood cardioplegia (Yau et al., 1993) can also result in the hypothermic heart being exposed to high levels of fatty acids.

Adverse Effects of Fatty Acids

Reperfusion of ischemic hearts in the presence of a high concentration of fatty acids is associated with a lower recovery of function and a decrease in cardiac efficiency (Lopaschuk et al., 1994a; Liedtke et al., 1988; Lopaschuk and Saddik, 1992). Whether the deleterious effect of fatty acids observed during ischemia-reperfusion are mediated by a similar mechanism during hypothermia-rewarming is unknown. Potential mechanisms proposed to explain the effects of fatty acids during ischemia-reperfusion include: 1) the disruptive effects of amphipathic metabolites of fatty acid metabolism on membrane function (Corr et al., 1984), 2) energy wastage via the futile cycling of triacylglycerol breakdown and re-synthesis (Van Der Vusse et al., 1992) and 3) a shift in myocardial metabolism away from carbohydrates towards fatty acid oxidation during reperfusion (Lopaschuk et al., 1994a). Although several studies have suggested that acyl-carnitine and acyl-CoA are capable of disrupting membrane function through their "detergent like" properties (Corr et al., 1984), more recent studies have dissociated the depression in heart function observed following ischemia-reperfusion from the build up of these metabolites (Lopaschuk et al., 1988; Heathers et al., 1993; Liedtke et al., 1988). Furthermore, hypothermia leads to a decrease in the levels of fatty acyl-CoA and acyl-carnitine (Mjos et al., 1991), suggesting that these metabolites do not play a role in mediating the deleterious effects of fatty acids during hypothermia-rewarming. The release of fatty acids from triacylglycerol stores and their subsequent ATP dependent re-incorporation back into triacylglycerol is thought to set up a futile cycle during ischemia, aggravating the depletion of ATP (Van Der Vusse et al., 1992). This aspect of fatty acid metabolism is unlikely to play an important role in mediating the effect of hypothermia as ATP levels are not depleted during hypothermia (Mjos et al., 1991, Steigen et al., 1994) and previous studies have shown that cooling rat hearts to 23° C leads to a greater than 5 fold reduction in the incorporation of fatty acids into triacylglycerol (Stein and Stein, 1963). The effect of fatty acids on glucose metabolism during hypothermia, and the subsequent effect of glucose metabolism on cardiac function were addressed in this thesis research.

The exact mechanism by which a fatty acid-induced depression of glucose oxidation affects functional recovery of the heart is unknown. Perfusing hearts with fatty acids, or increasing plasma fatty acid levels leads to an oxygen wasting effect (Vik-Mo and Mjos, 1981, Hutter et al., 1985; Challoner and Steinberg, 1966), in which more oxygen is consumed for a given level of cardiac power output. This effect is explained, in part, by the higher level of oxygen required to obtain the same amount of ATP from fatty acids (RQ = 0.7) than from carbohydrates (RQ = 1.0) (Opie, 1991). Under conditions where O_2 availability is limiting, this may limit maximal ATP production and depress heart function.

An alternate theory, is that fatty acids contribute to Ca^{2+} overload during ischemia-reperfusion which makes the heart inefficient, leads to a depression in heart function, and is a major factor leading to irreversible injury and cell necrosis. Stimulation of fatty acid oxidation depresses glucose oxidation to a greater extent than glycolysis. The net production of 2 ATP from glycolysis and its subsequent hydrolysis leads to the production of 2 H^+ when glycolysis is not coupled to glucose oxidation (Dennis et al., 1991). If glycolysis is fully coupled to glucose oxidation then net H^+ production from glucose metabolism is 0. An increased H^+ production can lead to a Ca^{2+} overload through the actions of the Na^+-H^+ and Na^+-Ca^{2+} exchangers (Tani and Neely, 1989). Stimulation of glucose oxidation leads to a tighter coupling of glycolysis and glucose oxidation, therefore reducing H^+ production under conditions such as ischemia-reperfusion (Lopaschuk et al., 1993).

Another theory to explain the adverse effects of fatty acids proposes that PDC activity is important to maintaining cytosolic phosphorylation potential. Increasing PDC activity is related to an enhancement of the cytosolic phosphorylation potential, improving the activity of ATP dependent reactions (Zweier and Jacobus, 1987; Bunger et al., 1989). The stimulation of PDC is thought to lead to an increase intramitochondrial NADH, allowing the myocyte to become more responsive to energy demand, since ATP formation by oxidative phosphorylation under these conditions is primarily driven by ADP availability (Zimmer et al., 1989; From et al., 1990). The activity of several important enzymes, including myofibrillar ATPase, the Na^+- K^+ ATPase and the sarcoplasmic reticulum Ca^{2+}-ATPase, are sensitive to changes in the cytosolic phosphorylation potential (Veech et al., 1980; Kammermeier et al., 1992; Mallet and Bunger, 1994). Therefore an increase in fatty acid oxidation at the expense of glucose oxidation could also adversely affect the activity of these enzymes and depress cardiac function.

Energy Substrate Metabolism and Hypothermia.

Although hypothermia is widely used as a means of suppressing energy demand and energy substrate metabolism, little is known about the utilization of specific energy substrates under hypothermic conditions. In whole animal studies, hypothermia appears to result in a decrease in glucose utilization despite an increase in glucose availability through increased plasma concentrations (Hoo-Paris et al., 1988; Jourdan, 1991; Tveita et al., 1996). In studies more specifically related to the heart, the measurement of arterio-venous differences in substrate composition across the heart in hypothermic subjects has not lead to a clear consensus regarding myocardial energy substrate metabolism under hypothermic conditions. While many studies have suggested that myocardial glucose metabolism is decreased during hypothermia, they have also suggested that the utilization of other carbohydrates such as pyruvate and lactate are increased relative to their normal euthermic rates (Shida et al., 1977; Teoh et al., 1988; Edwards et al., 1954). Some studies have suggested that overall carbohydrate metabolism is decreased, resulting in an increase in the reliance of fatty acid oxidation as a source of energy (Russ and Lee, 1965; Shida et al., 1977). These studies suffer from an inability to control the substrate composition of the blood in these hypothermic animals, and an inability to accurately account for the fate of substrates absorbed by the heart (i.e. metabolized or stored substrates will give the same response).

In addition to the uncertainty of substrate metabolism under hypothermic conditions, little is known about substrate metabolism in the rewarmed heart. Following rewarming, measurement of arterio-venous differences in energy substrate composition have suggested that carbohydrate oxidation may be depressed (Drake-Holland et al., 1984), increased (Teoh et al., 1988a), or both (Smolenski et al., 1994) depending on the timing of the sampling (decreased during the early phase and increased at later time points). Similarly, fatty acids oxidation may be increased (Mickle et al., 1986) or decreased (Teoh et al., 1988b). Again, the lack of a clear consensus leads to uncertainty over the fate of the energy substrates taken up by the heart.

The use of radioisotopically labeled substrates ($^3H/^{14}C$-glucose, ^3H-palmitate) in the present study provides a more direct approach to measuring energy substrate metabolism during hypothermia and following rewarming.

Cold-Tolerant vs Cold-Sensitive Species

From the earliest use of hypothermia as a protective element in heart surgery, researchers have suggested that hibernating species provide a good experimental model for avoiding the damaging effects of hypothermia (Bigalow et al., 1950; Hearse et al., 1981). The superior ability of hearts from hibernating species to tolerate low temperatures relative to cold-sensitive species (such as the rat) has been documented in a number of studies (Zimny and Taylor, 1965; Burlington et al., 1976; Caprette and Senturia, 1984; Burlington and Darvish, 1988, Burlington et al., 1989; Liu et al., 1996a). Despite the extensive evidence that low temperature physiology and biochemistry differs between the hearts of cold-tolerant and cold-sensitive species, little is known about how this relates to energy substrate metabolism in these species. During hibernation, cold-tolerant species, such as the Richardson's ground squirrel used in this study, rely primarily on fatty acids as an energy substrate (South and House, 1967). Analysis of energy substrate metabolism and function in ground squirrel hearts subjected to hypothermia-rewarming provides a model for comparison with rat hearts treated under the same conditions.

Controlling the Balance of Glucose and Fatty Acid Oxidation During Hypothermia.

As the balance between carbohydrate and fatty acid oxidation can affect the recovery of function in a number of pathological conditions (Lopaschuk et al., 1994a), an understanding of the factors controlling substrate metabolism is important for treatment aimed at improving functional recovery. In the heart, acetyl-CoA carboxylase plays a role in regulating fatty acid oxidation (Saddik et al., 1993; Awan and Saggerson, 1993) through the production of malonyl-CoA, a potent inhibitor of carnitine palmitoyltransferase 1 (McGarry et al., 1983; Cook, 1984). While acetyl-CoA carboxylase plays a role in regulating fatty acid oxidation under normothermic conditions, it is not known whether it plays a similar role under hypothermic conditions. As hibernating ground squirrels are more reliant on fatty acids as a source of energy than animals in the non-hibernating state, this should be reflected by changes in the activity of acetyl-CoA carboxylase activity between these two states.

Thesis Objectives

This thesis tested the hypothesis that a stimulation of glucose oxidation is beneficial to improving the recovery of function following hypothermia-rewarming in the presence of high concentrations of fatty acids. Isolated working hearts were used to compare hypothermic with normothermic conditions, under conditions which avoided the complicating factors of ischemia and cardioplegia, both of which can independently alter metabolism and function in the heart (Lopaschuk et al., 1994a; Kupriyanov et al., 1995). To determine how fatty acids effect glucose metabolism under hypothermic conditions, rat hearts were perfused with or without 1.2 mM palmitate. To examine how hypothermia-rewarming affects energy substrate metabolism and function, these parameters were examined in rat hearts (cold-sensitive) and ground squirrel hearts (cold-tolerant). To examine the role of Ca^{2+} in mediating the effects of hypothermia-rewarming on heart function, hearts were perfused with high or low concentrations of Ca^{2+}. To determine if direct stimulation of glucose oxidation is beneficial in improving functional recovery, hearts were perfused with or without the PDC agonist dichloroacetate. To determine if ACC could play a role in regulating fatty acid oxidation under hypothermic conditions, fatty acid oxidation and ACC activity were examined in hearts isolated from ground squirrels in the hibernating or the non-hibernating state.

Effects of High Levels of Fatty Acids on Glucose Metabolism during and Following Hypothermia.

If the effect of fatty acids in depressing the recovery of function following hypothermia-rewarming is based on a depression of glucose metabolism, then glucose metabolism should be depressed under hypothermic as well as normothermic conditions. As described in Chapter III, the addition of high levels of fatty acids (1.2 mM palmitate) to the perfusion medium exacerbated the recovery of function in rat hearts following hypothermia-rewarming. Our results, support the observations made by Mjos et al (1991) and Steigen et al (1994) that fatty acids are detrimental to the recovery of function. By directly measuring glucose metabolism (glycolysis and glucose oxidation) we demonstrated that the addition of 1.2 mM palmitate to the perfusion medium depresses glucose metabolism during hypothermia and following rewarming. As anticipated, palmitate led to a greater depression in glucose oxidation than glycolysis, an effect which is also observed under normothermic conditions (Lopaschuk et al., 1993; Schonekess et al., 1995). Since this effect was observed under normothermic and hypothermic conditions, we demonstrate that palmitate retains the ability to depress glucose oxidation at both 15° C and 37° C. Previous estimates of hypothermia on glycolysis (determined by measuring lactate production and glycogen depletion) have also suggested that glycolysis is greatly suppressed by hypothermia (Orme et al., 1977). By perfusing hearts with ^{3}H-glucose and relevant concentrations of fatty acids, we were able to support this conclusion by directly measuring the flux of glucose through the glycolytic pathway.

Although hypothermia led to a depression in glucose metabolism, it did not affect glycolysis and glucose oxidation equally. Whether the hearts were perfused with palmitate or not, cooling to 15° C led to a greater inhibition of glycolysis than glucose oxidation. The fact that palmitate retained the ability to depress glycolysis during hypothermia suggests that low temperature inhibition of an enzyme, or enzymes, in the glycolytic pathway is not absolutely limiting the rate of glycolysis. As glycolytically derived ATP is thought to be preferentially used for ion homeostasis (Kristensen 1993; Glitsch and Tappe, 1993; Xu et al., 1995; Paul et al., 1989), it is tempting to speculate that the depression of glycolysis during hypothermia is generally responsible for the loss of ion homeostasis during hypothermia, and that the further decrease in glycolysis by high concentrations of fatty acids aggravates this effect. However, as later studies revealed a similar decrease in glycolysis in ground squirrel hearts during hypothermia without any depression in function following rewarming, it is unlikely that the amount of glycolytically derived ATP during hypothermia is a determinant of functional recovery between the two species. (This does not, however, preclude the possibility that glycolytically derived ATP is used differently within the species during hypothermia).

Previous studies based on whole animal hypothermia (Hoo-paris et al., 1988; Tveita et al., 1996), and the measurement of arterio-venous differences in energy substrate concentrations across the heart (Shida et al., 1977, Russ and Lee, 1965), have suggested that glucose metabolism is generally depressed in the whole body as a result of hypothermia, and that this effect occurs within the heart as well. The data presented in this thesis supports these observations, but suggests that this is more likely an effect of glycolysis rather than carbohydrate oxidation.

Energy Substrate Metabolism in Rats and Ground Squirrels.

Measurement of function and energy substrate metabolism in rat and ground squirrel hearts under the hypothermia-rewarming protocol provide a means of comparing values obtained from cold-sensitive and cold tolerant-species. Cold-tolerant species, such as ground squirrels, have adapted to utilizing fatty acids as a source of energy during hibernation (Willis, 1982), and the performance of ground squirrel hearts under hypothermic conditions in the presence of high levels of fatty acids is better than that observed in rat hearts (Burlington and Darvish, 1988).

Early investigators working on the hypothermic preservation of heart tissue suggested that hibernating species provide an experimental model for research aimed at avoiding the damaging effects of hypothermia (Bigalow et al., 1950, Hearse et al., 1981). We therefore compared energy metabolism and mechanical function in ground squirrels and rats to determine if species differences in metabolism exist which may account for their differences in cold tolerance (Chapter IV).

In rat hearts perfused with high concentrations of fatty acids (1.2 mM palmitate and 1.25 mM Ca^{2+}) cardiac power output following rewarming was significantly lower than the level obtained during the pre-hypothermic control period. In contrast to rat hearts, ground squirrel hearts demonstrated a complete recovery of function following rewarming, despite the presence of high levels of fatty acids. Although the level of cardiac power output in rat hearts was significantly lower than in ground squirrel hearts over the hypothermic period, power output in both species was constant over the 2 hr period of hypothermia.

Hypothermia is capable of altering the pattern of energy substrate metabolism either directly, through inhibition of the rate at which enzymes catalyze reactions, or by a reduction in overall metabolic demand through a decrease in the work being performed by the heart. As a result, general changes in energy substrate metabolism, as well as work related changes in metabolism were examined. Analysis of energy substrate metabolism revealed the same general response to hypothermia-rewarming in both species, namely that glycolysis was depressed to a greater extent than oxidative metabolism, and that rate of glucose oxidation was less depressed than the rate of palmitate oxidation during hypothermia. The similar general pattern of substrate metabolism in the two species suggests that species differences cannot be explained by differences in the sensitivity of the energy yielding pathways to depression by hypothermia. For example, although glycolytically derived ATP is thought to be preferentially used for ion homeostasis (Kristensen 1993; Xu et al., 1995), our results suggest that the level of ATP production from glycolysis during hypothermia cannot explain why ground squirrels are better than rats at maintaining function at low temperatures (Liu et al., 1991; Liu and Wang, 1993; Liu et al., 1996a).

Previous studies have suggested that fatty acid oxidation is increased during hypothermia as a result of a depression in carbohydrate metabolism (Russ and lee, 1965; Shida et al., 1977; Jourdan , 1991; Steigen et al., 1993). The results obtained in Chapter IV show that although oxidative metabolism assumes a greater role in energy metabolism during hypothermia, hypothermia itself does not lead to an obligatory increase in the proportion of energy derived from fatty acid oxidation at the expense of glucose oxidation. Our results show that although fatty acids are capable of depressing glucose oxidation under hypothermic conditions, this effect is not compounded by differences in the thermal sensitivity of carbohydrate and fatty acid oxidation which would favor fatty acid oxidation. In fact, the increased contribution of glucose oxidation to energy production under hypothermic conditions suggests that modification of PDC activity to improve carbohydrate oxidation is possible under these conditions.

Although the general pattern of energy substrate metabolism was similar for rats and ground squirrels, species differences did appear when energy substrate metabolism was normalized for cardiac power output. Oxidative metabolism provided the majority of ATP for the heart under either normothermic or hypothermic conditions (>95% based on maximum theoretical ATP production).

and provides the most important source of energy for contractile work (Opie, 1991). As substrate metabolism is driven by energy demand, as well as the intrinsic activity of the various pathways, oxidative metabolism was also examined relative to the power output of the heart. The increased contribution of glucose oxidation to cardiac power output observed in both species suggests that this is a universal effect of hypothermia. That it occurs in both species when the supply of pyruvate from glycolysis is dramatically reduced implies a specific activation of PDC. It may be related to a rise in intramitochondrial Ca^{2+} during hypothermia, leading to activation of the PDC through Ca^{2+}-mediated activation of pyruvate dehydrogenase phosphatase (McCormack et al., 1992).

In contrast to glucose oxidation, palmitate oxidation differed between the species in response to hypothermia. In ground squirrel hearts, the increased reliance on glucose oxidation was accompanied by a decrease in the contribution from palmitate. In rat hearts, this effect did not occur, and palmitate oxidation normalized for cardiac power actually increased slightly. Under normal conditions, an increased reliance on glucose results in a corresponding decrease in fatty acid utilization (Randle et al., 1994, Sugden and Holness, 1994). Under hypothermic conditions, our data suggest that either this reciprocal regulation of substrate metabolism does not function at low temperature, or that the demand for energy for non-work related processes (i.e. ion homeostasis) is increased. An increased energy demand could be met under hypothermic conditions by increasing the contribution of palmitate to energy production. As palmitate metabolism accounts for the bulk of acetyl-CoA production in these heart, these species differences led to a large difference in the efficiency of converting energy into cardiac power under hypothermic conditions.

The species differences in cardiac efficiency as a result of hypothermia were confirmed by a separate series of experiments in which O_2 consumption was measured at 37° C and 15° C. In both species O_2 consumption and cardiac power decreased upon cooling. However, the disparity between O_2 consumption and cardiac power output between the two resulted in an increased cardiac efficiency in ground squirrels and a decreased efficiency in rats. The increased cardiac efficiency measured in ground squirrels using O_2 consumption as a metabolic index is similar to the values reported by Burlington and Darvish (1988) under similar conditions using a different species of ground squirrel (*Spermophilus tridecemlineatus*) than the one used in our study. This suggests that an increase in cardiac efficiency during hypothermia is an intrinsic aspect of the relationship between function and metabolism in ground squirrels. The effect of temperature on cardiac efficiency in cold-sensitive species is variable according to the species and the extent of cooling. Some studies have shown that a mild hypothermia of only a few degrees can increase cardiac efficiency in dogs (Monroe et al., 1964) and rats (Burlington and Darvish, 1988), while deeper hypothermia may lead to a reduction cardiac efficiency in dog hearts (Archie and Kirklin, 1973), and rat myocardial tissue (Loiselle, 1979). This species difference in the ability to couple energy substrate metabolism to cardiac power output could possibly limit the recovery of function following rewarming. The poor recovery of function following rewarming coupled with a decrease in cardiac efficiency is similar to the myocardial stunning obtained following ischemia-reperfusion (DeMaison and Grynberg, 1994; Hata et al., 1994; Laster et al., 1989; Kusuoka et al., 1990). The species difference observed in our study raises the question of whether the poor recovery of function observed in rat hearts following rewarming has its origin in rewarming, or whether it begins during the hypothermic perfusion period.

H^+ production, as a result of the imbalance between glycolysis and glucose oxidation, as proposed for ischemia-reperfusion (Lopaschuk et al., 1993), could potentially be responsible for the poor recovery of function following hypothermia-rewarming. However, our results suggest that it is unlikely that H^+ production during hypothermia plays a significant role in mediating the poor recovery in rat hearts. This is because of the dramatic reduction in H^+ production which occurs in both species as a result of the greater depression of glycolysis than glucose oxidation during hypothermia. As a result, increased H^+ production during hypothermia cannot explain the higher level of cytosolic Ca^{2+} observed in isolated rat cardiac myocytes relative to ground squirrel cardiac myocytes (Liu et al., 1991, Liu and Wang, 1993). Similarly, unlike reperfusion following ischemia (Lopaschuk et al., 1993), H^+ production was not markedly enhanced during the rewarmed period. The only species difference which may account for the effects of fatty acids occurred during rewarming itself, when increased glycolysis in rat hearts led to a significantly higher rate of H^+ production. This may explain the observation by Mjos et al (1991) that fatty acids are most detrimental when present during rewarming itself, and the observation by Steigen et al (1994), that rewarming rat hearts in the presence of 1.2 mM palmitate is associated with a higher level of tissue Ca^{2+} compared to hearts rewarmed in the absence of palmitate. An overproduction of H^+ during rewarming may have prevented or slowed the re-establishment of a normal intracellular Ca^{2+} concentration in rat hearts during rewarming when they may be trying to unload excess Ca^{2+} accumulated during hypothermia. A lower H^+ production in ground squirrels during rewarming, along with better control of intracellular Ca^{2+} during hypothermia (Liu et al., 1991; Liu and Wang, 1993, Liu et al., 1996a), may have contributed to the better recovery of function following rewarming in this species.

The Effects of Ca^{2+} *in Mediating the Recovery of Function of Hypothermic Hearts.*

An intracellular Ca^{2+} overload has been proposed to play a role in mediating the damaging effects of ischemia-reperfusion (Tani and Neely, 1989) and hypothermic storage (Stringham et al., 1992; Robinson and Harwood, 1991). The susceptibility of the hypothermic heart to Ca^{2+} overload is thought to be related to a reduction in the Na^+-K^+ ATPase during hypothermia leading to a rise in intracellular Na^+ which, through the actions of the Na^+-Ca^{2+} exchanger, leads to an increased level of intracellular Ca^{2+} (Martineau-Knerr and Lieberman, 1993). This effect is exacerbated by the fact that the Na^+-Ca^{2+} exchanger is less sensitive to inhibition by low temperature than the Na^+-K^+ ATPase (Shattock and Bers, 1987). Similarly, hypothermia can result in a loss of sarcoplasmic reticulum function (Labow et al., 1993; Fukumoto et al., 1991, Liu et al., 1993, 1994) which may contribute to the loss of Ca^{2+} homeostasis and the reduction in heart function. Hypothermia in the presence of high levels of fatty acids leads to a higher myocardial tissue level of Ca^{2+} which is thought to contribute to the poor recovery of function following rewarming (Steigen et al., 1994, Aasum and Larsen, 1996). Despite the proposed role of increased myocardial Ca^{2+} in mediating the poor recovery following rewarming (Steigen et al., 1994), and its role in mediating a reduction in cardiac efficiency (DeMaison and Grynberg, 1994; Kusuoka et al., 1990), Ca^{2+} is capable of stimulating glucose oxidation (Schonekess et al., 1995) secondary to a stimulation of mitochondrial dehydrogenases (McCormack and Denton, 1989). As a result, Ca^{2+} can potentially exert both beneficial and detrimental effects on the heart during hypothermia and following rewarming in the presence of fatty acids.

In order to examine this effect, cardiac function and energy substrate metabolism was examined in hearts perfused with either a high (2.50 mM) or a low (1.25 mM) Ca^{2+} concentration. Ground squirrel hearts, which are unaffected by the damaging effects of hypothermia, and which demonstrate a better capability of regulating intracellular Ca^{2+} at low temperatures (Liu et al., 1996a; Liu and Wang, 1993), were also examined for comparison purposes.

In rat hearts, increasing perfusate Ca^{2+} had the paradoxical effect depressing cardiac power output during hypothermia, but improving the recovery of function following rewarming. That increasing perfusate Ca^{2+} from 1.25 mM to 2.50 mM did not affect any of the functional parameters during the initial 37° C perfusion period confirms previous reports that changes in Ca^{2+} over this concentration range does not result in a positive inotropic effect (Bielefeld et al., 1983; Shonekess et al., 1995). This suggests that the differences in function observed following rewarming at the different Ca^{2+} concentration are not due to inotropic actions of Ca^{2+}, but rather due to direct actions of Ca^{2+} on the hypothermia-rewarming protocol on these hearts.

The general effect of increasing perfusate Ca^{2+} was a stimulation of glucose oxidation and a reduction in palmitate oxidation in rat hearts. The increase in glucose oxidation is likely the result of an increase in mitochondrial Ca^{2+} levels leading to activation of the pyruvate dehydrogenase complex (PDC)(McCormack and Denton, 1989). The increased cardiac power output obtained in rat hearts perfused with the high Ca^{2+} concentration following rewarming may also have contributed to the high level of glucose oxidation in these hearts by stimulating metabolic demand. The action of Ca^{2+} in stimulating glucose metabolism in working rat hearts is similar to recently published observations obtained under normothermic conditions (Shonekess et al., 1995). The stimulation of glucose oxidation in rat hearts led to a reciprocal decrease in palmitate oxidation, suggesting that reciprocal control of carbohydrate and fatty acid oxidation (Randle et al., 1994, Sugden and Holness, 1994) does occur under hypothermic conditions despite the decrease in overall metabolism caused by hypothermia. Despite this general effect, increasing perfusate Ca^{2+} increased the individual contribution of glucose and palmitate oxidation to cardiac power output in rat hearts, indicating that a greater degree of uncoupling between energy substrate metabolism and cardiac power had occurred. These results suggest that raising perfusate Ca^{2+} led to an increase in tissue Ca^{2+} resulting in both a stimulation of glucose oxidation and a reduction in cardiac efficiency.

As anticipated, raising perfusate Ca^{2+} concentration only had a minimal effect on glucose metabolism in ground squirrel hearts, and did not affect the contribution of glucose and palmitate oxidation towards cardiac power output under hypothermic conditions. Regardless of perfusate Ca^{2+} concentration, cardiac efficiency increased in ground squirrel hearts during hypothermia The ability of ground squirrel hearts to regulate intracellular Ca^{2+}, and prevent a decrease in cardiac efficiency, under hypothermic conditions (Liu and Wang, 1993; Liu et al., 1996a) provides further evidence that Ca^{2+} plays a role in determining cardiac efficiency under hypothermic conditions.

Despite the observation that increasing perfusate Ca^{2+} depressed cardiac power output and decreased efficiency in rat hearts during hypothermia, this effect did not translate into a poorer recovery of function following rewarming. This suggests that the recovery of function following rewarming occurs independently of cardiac power and efficiency during hypothermia and suggests that cardiac efficiency during hypothermia is not the sole determinant of functional recovery following rewarming. Although hypothermia leads to a rise in myocardial tissue Ca^{2+} levels (Steigen et al., 1994, Aasum and Larsen, 1996), these results imply that Ca^{2+} itself is not directly correlated with the poor recovery of function following rewarming, but rather that the recovery of function depends on other factors. This assessment is in agreement with results of Aasum and Larsen (1996). These authors showed that hypothermia itself results in a significant rise in tissue Ca^{2+} levels, but that the recovery of function following rewarming is more closely correlated with a stimulation of glucose oxidation. In the present study, the improved functional recovery following rewarming in the 2.50 mM Ca^{2+} group was also associated with an increased glucose oxidation.

In hearts subjected to ischemia-reperfusion, the stimulation of glucose oxidation leads to a significant reduction in H^+ production from glycolysis uncoupled from glucose oxidation (Lopaschuk et al., 1993). In this study, the stimulation of glucose oxidation was not accompanied by a reduction in H^+ production between the groups. Hypothermia led to a significant reduction in H^+ production regardless of perfusate Ca^{2+} concentration. The only major difference between the groups was noted during the period of rewarming. Glucose oxidation was higher during rewarming in rat hearts perfused with 2.50 mM Ca^{2+}, than in hearts perfused with 1.25 mM Ca^{2+}, leading to a H^+ production which was significantly lower than that observed during the initial 37° C perfusion period. This would support the argument that the damaging effects of fatty acids are most prevalent during rewarming (Mjos et al., 1991; Steigen et al., 1994). However, the high perfusate Ca^{2+} concentration, and the fact that H^+ production is still significantly higher in rats than ground squirrels, suggests that H^+ production alone does not determine the recovery of function during rewarming.

Recent studies have also suggested that substrates which increase PDC activity, such as pyruvate, makes the myocyte more responsive to metabolic demand (From et al., 1990), and leads to an increase in cytosolic phosphorylation potential which has been shown to be beneficial in improving Ca^{2+} handling by sarcoplasmic reticulum (Mallet and Bunger, 1994). This may be beneficial in heart tissue where sarcoplasmic reticulum function is damaged as a result of hypothermia (Labow et al., 1993, Belke, Jourdan and Wang, unpublished observations). It is conceivable that sarcoplasmic reticulum function is decreased in rat hearts following hypothermia, and that the increase in glucose oxidation in hearts perfused with 2.50 mM Ca^{2+} was beneficial in improving excitation-contraction coupling in these hearts.

Direct Stimulation of PDC by Dichloroacetate in the Hypothermic-Rewarmed Heart.

Perfusing hearts with high concentrations of fatty acids leads to a depression of glucose oxidation, while increasing perfusate Ca^{2+} concentration leads to a stimulation of glucose oxidation. Increasing perfusate Ca^{2+}, however, may have a number of effects on myocardial physiology apart from an increase in glucose oxidation. Therefore, we wanted to determine if a selective stimulation of glucose oxidation was beneficial to the recovery of function following hypothermia-rewarming. To determine if increasing glucose oxidation is beneficial during hypothermia-rewarming, rat hearts were perfused with 1 mM DCA to stimulate PDC activity. Following ischemia, DCA has been shown to stimulate glucose oxidation, reduce H^+ production and improve functional recovery (McVeigh and Lopaschuk, 1990; Lopaschuk et al., 1993, Lewendowski and White, 1995), and improve cardiac efficiency in congestive heart failure

(Bersin et al., 1994). DCA offers the advantage of directly stimulating glucose oxidation without the complications arising from the increased perfusate Ca^{2+} in the previous study. DCA stimulates glucose oxidation by inhibiting the actions of PDH kinase, thereby preventing the phosphorylation and inactivation of the PDC (Stacpoole, 1989). To determine if the stimulation of glucose oxidation over the course of the perfusion or just prior to rewarming was beneficial to the recovery of function following rewarming, 1 mM DCA was added at the beginning of the perfusion protocol, or just prior to rewarming.

The addition of DCA to the perfusion medium led to a significant increase in glucose oxidation relative to untreated hearts. However, DCA was only beneficial in improving the recovery of function if added at the beginning of the experiment, and was not beneficial if added just prior to rewarming. As a result, hypothermia-rewarming differs from ischemia-reperfusion where DCA is only beneficial if added during reperfusion, and can actually be detrimental if added prior to ischemia (McVeigh and Lopaschuk, 1990). In ischemia-reperfusion, the detrimental effects of DCA added prior to ischemia are thought to be related to an increased H^+ production due to an increased uncoupling of glycolysis from glucose oxidation. As the hearts in the present study were constantly perfused, the mild stimulation of glycolysis caused by DCA was not detrimental since any H^+ produced could be readily removed from the heart.

As shown in Chapter VI, the stimulation of glucose oxidation only led to a significant improvement in cardiac function following rewarming if DCA was present throughout the perfusion protocol. DCA's effects on H^+ production during rewarming, were only evident due to a faster recovery of glucose oxidation than glycolysis. However, the addition of DCA just prior to rewarming also depressed H^+ production, but did not lead to a significant improvement in function following rewarming. This suggests that H^+ production during rewarming is not the sole determinant of functional recovery that can be attributed to the stimulation of glucose oxidation.

DCA was also beneficial in improving cardiac efficiency during the hypothermic and rewarmed periods when added to the perfusion medium at the beginning of the experiment. Previous studies have shown that DCA can improve cardiac efficiency following ischemia-reperfusion (Liu et al., 1996b; Lewendowski and White, 1995), or in patients with congestive heart failure (Bersin et al., 1994). In our studies, the increase in efficiency during hypothermia was due to a slight decrease in palmitate oxidation relative to cardiac power output. This suggests that DCA reduced non-work related energy expenditure during hypothermia. Recent work by Terje Larsen's group has shown that the accumulation of Ca^{2+} in rat hearts during hypothermia is lower in hearts treated with DCA (Terje Larsen - personal communication). This supports the idea that tissue Ca^{2+} is responsible for determining cardiac efficiency at low temperatures.

The addition of DCA pre-hypothermia led to a significant increase in glucose oxidation relative to untreated hearts over the course of the perfusion protocol. The stimulation of glucose oxidation led to a decrease in palmitate oxidation which was significant during the hypothermic perfusion period. The stimulation of glucose oxidation by DCA is thought to inhibit palmitate oxidation by providing more acetyl-CoA for the production of malonyl-CoA (a potent inhibitor of CPT-1) by acetyl-CoA carboxylase (ACC) (Saddik et al., 1993). This occurs because acetyl-CoA derived from PDC is very accessible to carnitine acetyl-transferase and a transport system to remove it from the mitochondrial, while acetyl-CoA derived from β-oxidation is not (Lysiak et al., 1986). As a result, DCA not only stimulates glucose oxidation, but it also increases the contribution of glucose oxidation to overall energy metabolism, since it decreases palmitate oxidation (Lopaschuk and Saddik, 1992). That this effect is observed during hypothermia, suggests that this mechanism for regulating the contribution of palmitate to energy substrate metabolism is unaffected by low temperature. The increased contribution of glucose oxidation to overall energy metabolism following DCA administration is similar to that observed when perfusate Ca^{2+} is increased. These results suggest that CPT 1 retains its role in regulating fatty acid oxidation under hypothermic conditions, and that the activity of regulatory enzymes such as ACC continue to play a role in regulating fatty acid oxidation during and following hypothermia. As a result, pharmacologically manipulating ACC may be an approach to altering the contribution of glucose and fatty acids to ATP production during hypothermia and rewarming.

ACC Activity Regulates Palmitate Oxidation in Hibernating Ground Squirrels.

If ACC plays a role in regulating fatty acid oxidation under hypothermic conditions, then its activity should be decreased in hibernating animals to facilitate fatty acid utilization during hibernation. Hibernating species increase body fat content prior to entry into hibernation, and rely on fatty acid mobilization from adipocyte triacylglycerol stores as a source of energy during the hibernating phase (Lyman and Chatfield, 1955). The majority of studies on fatty acid metabolism in hibernating species have dealt with fatty acid synthesis and storage (Willis 1982), as well as seasonal variations in fat mobilization (Florant et al., 1993). However, little attention has been paid to fatty acid oxidation by the tissues apart from its importance in non-shivering thermogenesis by the brown adipose tissue (Nedergaard et al., 1989). In this study, glucose and fatty acid oxidation was examined in isolated working hearts from non-hibernating and hibernating ground squirrels. Hearts were perfused at both 37 and 5°C; temperatures which are observed under active and hibernating conditions. Hibernating animals used in the present study were aroused from hibernation prior to removal of the heart to avoid differences in metabolism which might arise as a result of starting heart temperature, or being in hibernation at the time of collection. Therefore, differences in substrate metabolism between the groups in the study presented in Chapter VII are indicative of physiological changes due to being in the hibernating phase and not body temperature *per se*. Our data suggest that hearts from animals in the hibernating phase have a greater reliance on fatty acids as a source of energy, with the greatest differences being observed at low temperature. It should be noted that these differences were seen even though hearts were perfused with similar concentrations of fatty acids. Because circulating levels of fatty acids are elevated in hibernating animals compared to non-hibernating animals (Galster and Morrison, 1966) it is likely that this shift towards the preferential use of fatty acids is even greater *in vivo*. Furthermore, the increase in the contribution of fatty acid oxidation to TCA cycle activity in hibernating ground squirrel hearts under perfusion conditions identical to those used for the non-hibernating hearts, demonstrates that the heart muscle itself directly adapts to the hibernating state.

Previous studies have indirectly suggested that the hearts of hibernators are more reliant on fatty acid oxidation as a source of energy, based upon increased carnitine content (Burlington and Shug, 1981), and a decrease in the activity of key regulatory enzymes involved in glycolysis and glucose oxidation (Brooks and Story, 1992). However, increased tissue carnitine content is not a good indicator of fatty acid oxidation, as carnitine can also regulate glucose metabolism, secondary to its effects on carnitine acetyltransferase activity (Broderick et al., 1992). Similarly, the decrease in glucose metabolism suggested by Brooks and Story (1992) was thought to occur during hibernation as a means of suppressing metabolic rate; however, these authors did not address the contribution of non-carbohydrates to energy metabolism. In this study we observed that the rate of glucose oxidation in hearts from hibernators was lower than non-hibernators, but that glucose oxidation was affected similarly by a decrease in temperature in both groups.

When O_2 and substrate supplies are not limiting, overall oxidative metabolism is determined by the metabolic demand, with the balance between carbohydrate and fatty acid oxidation being determined by the activity of the pyruvate dehydrogenase complex (PDC) and CPT 1 (Sugden and Holness, 1994; Randle et al., 1994; Lopaschuk et al., 1994a). The balance of substrate entry into the heart is regulated by reciprocal inhibition between the two pathways. Although the control exerted by fatty acid oxidation on PDC activity through phosphorylation is well known (see Patel and Roche, 1990 for review), the reciprocal effect of carbohydrate metabolism on fatty acid oxidation is less understood. Recent studies have suggested that fatty acid oxidation in the heart is regulated by the activity of CPT 1 (Saddik et al., 1993, Awan and Saggerson, 1993). This enzyme is inhibited by malonyl-CoA, a product of ACC, which is used in the synthesis of fatty acid. CPT 1 in heart tissue is extremely sensitive to inhibition by malonyl-CoA (I_{50} = 30-80 nM) (Mills et al., 1983; McGarry et al., 1980), and the recent discovery that ACC is expressed in the heart (Bianchi et al., 1990; Thampy, 1989) suggests a role for this enzyme in regulating fatty acid oxidation. A good correlation between the activity of ACC, tissue levels of malonyl-CoA and the rate of fatty acid oxidation has been observed under normal physiological conditions (Saddik et al., 1993) as well as under pathological conditions such as ischemia-reperfusion (Kudo et al., 1995, 1996).

In this study, the increased contribution of palmitate to overall oxidative metabolism in the hibernating group was associated with a decreased expression of the predominate ACC 280 isoform. This was accompanied by a decrease in ACC activity, regardless of whether activity was measured at 37 or 5° C. Malonyl-CoA levels in hearts frozen at the end of the 5° C perfusion also suggest that ACC activity was lower in the hibernating group, although malonyl-CoA levels did not differ significantly between hearts frozen at 37° C. Tissue levels of malonyl-CoA, however, are not an accurate index of cytosolic ACC activity as malonyl-CoA can also be produced in the mitochondria as a result of the non-specific use of acetyl-CoA by mitochondrial propionyl-CoA carboxylase (Scholte, 1969). The actual concentration of cytosolic malonyl-CoA available to inhibit CPT 1 in these hearts could not be accurately determined in this study. Our recent studies, however, have shown a good inverse relationship between measurements of cytosolic ACC activity in heart extracts and fatty acid oxidation rates (Kudo et al., 1995, 1996). A similar inverse correlation between ACC activity and fatty acid oxidation was also observed in this study.

It appears that the alteration in fatty acid oxidation in the hibernating ground squirrel hearts occurs as a result of an alteration in the expression of ACC 280. Recent evidence suggests that ACC 280 and 265 may be the products of separate genes (Abu-Elheiga et al., 1995). This may explain why the ACC 280 isoform is decreased in hibernating ground squirrels while the 265 isoform is unchanged. Since hibernators reduce their food intake prior to hibernation, the reduction in ACC 280 may be viewed as a response to fasting. However, preliminary results have shown that ACC 280 expression increases in brown adipose tissue during hibernation, (Belke, Wang and Lopaschuk; unpublished observations) making simple regulation of ACC 280 by fasting unlikely. However, additional experiments on animals in the "weight gain" phase of the hibernation cycle should be performed to clarify the relationship between food intake and ACC expression in this species. Although the activity of key enzymes involved in glucose metabolism are down-regulated during hibernation, it appears that changes in ACC expression and activity may also occur to promote the use of fatty acids as a fuel. This suggests that ACC activity may play an important role in determining substrate preference at low temperatures.

Conclusions

This thesis examined the effect of high concentrations of fatty acids on cardiac function and metabolism during and following hypothermia. This study tested the hypothesis that fatty acids depressed glucose metabolism, specifically glucose oxidation, during hypothermia and rewarming and that this effect depresses the recovery of function following rewarming. To test this hypothesis, cardiac function and metabolism were examined in hearts perfused in the presence or absence of high concentrations of fatty acids, and under conditions which stimulate glucose oxidation. To gain a better understanding of how metabolism was affected by hypothermia and rewarming, hearts from cold-sensitive species were compared with hearts from cold-tolerant species. This course of experiments determined that: 1) Perfusing hypothermic hearts with 1.2 mM palmitate led to a depression of glucose metabolism associated with a poorer recovery of function following rewarming. 2) The poor recovery of function does not appear to be the result of a severe reduction in glycolysis during hypothermia, but was more closely related to the level of glucose oxidation. 3) Glycolysis was similarly depressed in cold-tolerant species, but this did not affect the recovery of function following rewarming. 4) The efficiency of the heart under hypothermic conditions does not directly affect the recovery of function following rewarming in hearts from cold-sensitive species. 5) Stimulating glucose oxidation at the expense of palmitate (by increasing perfusate Ca^{2+} concentration or by the use of dichloroacetate) is beneficial in improving the recovery of function following rewarming. 6) Although the stimulation of glucose oxidation is important in reducing H^+ production in (no-flow) ischemia-reperfusion, H^+ production does not play a major role in mediating the effect observed following hypothermia-rewarming. This suggests that some other aspect of glucose oxidation plays a role in mediating this effect, possibly the role of pyruvate dehydrogenase complex activity in regulating the coupling between energy production and demand. 7) The activity of acetyl-CoA carboxlylase appears to play a role in regulating the balance of carbohydrate and fatty acid oxidation in hypothermic hearts. Pharmacological treatment aimed at increasing glucose oxidation and decreasing palmitate oxidation, possibly through the stimulation of acetyl-CoA carboxylase, could be beneficial in avoiding the damaging effect of increased fatty acid levels under hypothermic conditions.

These results support the hypothesis that fatty acids lead to a depression in glucose oxidation during hypothermia and contributes to the poor recovery of function following rewarming. Furthermore, a stimulation of glucose oxidation in the hypothermic heart is associated with a better recovery of function following rewarming. Acetyl-CoA carboxylase plays a role in regulating the balance of glucose and fatty acid oxidation in the heart under normothermic and hypothermic conditions. Pharmacological interventions aimed at shifting the balance of energy substrate metabolism away from fatty acid oxidation and towards glucose oxidation will be beneficial in alleviating the detrimental effects that high concentrations of fatty acids have on heart function under conditions of hypothermia-rewarming.

Limitations of Thesis Study

One of the limitations of the work presented in this thesis was the lack of a matched normothermic control perfusion. Although values obtained during the rewarmed period were compared with pre-hypothermic control period values, it was not possible to account for changes which may have occurred as a result of the length of the perfusion protocol. The controversy over using time matched controls for hypothermic perfusions stems from the fact that the two preparations are not metabolically equivalent. As a result, any changes in activity which occur in normothermic controls at a given time point may not correspond to changes which would be expected in the hypothermic preparation. Erroneous conclusions can be drawn from the hypothermic preparation on the basis of changes occurring in normothermic controls at the same time point.

Another limitation of the study stems from the fact that we did not actually measure tissue Ca^{2+} levels in our hearts under hypothermic and rewarmed conditions. However, the stimulation of glucose oxidation in rat hearts, but not ground squirrel hearts, suggest that increasing perfusate Ca^{2+} concentrations did at least raise mitochondrial Ca^{2+} concentrations to stimulate PDC. Similarly, the depressed cardiac function, and the decreased cardiac efficiency, are indicative of Ca^{2+} overload, suggesting an increased cytosolic Ca^{2+} concentration in the rat myocardium.

It was not possible in the present study to distinguish between a decrease in cardiac efficiency which occurs as a result of increased energy expenditure for non-work related ATP consuming reactions, vs an uncoupling of oxidative phosphorylation at the level of the mitochondria. Both scenarios would lead to an increase in substrate oxidation relative to the level of work obtained from the heart. Both scenarios may be active in the heart under normothermic and hypothermic conditions at the same time as neither effect is mutually exclusive of the other. It is unknown whether the relative coupling of oxidative phosphorylation in the mitochondria is similar between species, and whether this is affected differently in the species as a result of hypothermia and rewarming.

Due to the fact that the O_2 electrode is sensitive to temperature changes, it was not possible to examine oxidative metabolism during the period of rewarming to determine if an increase in cardiac power output during the rewarming phase outpaced O_2 consumption. This would have allowed us to determine if oxidative metabolism lagged behind energy demand during any part of the rewarming phase. An increase in cardiac power output is normally met by an increase in oxidative metabolism under normothermic conditions; however, it was not possible to determine if this occurs during rewarming as well. Examination of general metabolism by energy substrate metabolism does not allow for a fine enough resolution of minute by minute changes in energy metabolism over a short time period.

The conditions used in this study were chosen to specifically examine the effects of hypothermia on metabolism and function in the presence of high concentrations of fatty acids. Under clinical conditions, hypothermia is used in conjunction with cardioplegia, and the heart is exposed to periods of ischemia.

The effect of these additional factors on energy substrate metabolism and heart function must be examined before any valid conclusions regarding the clinical relevance of the effect of high concentrations of fatty acids on the hypothermic heart can be drawn.

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